BIOSURFACTANT PRODUCING BACTERIA FROM THE SELECTED SOILS OF KERALA

By

REMYA V. M.

THESIS

Submitted in partial fulfilment of the requirement for the degree of

Master of Science in Agriculture

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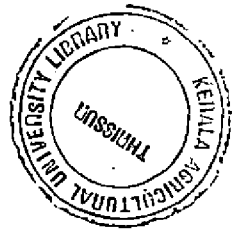

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2007

DECLARATION

I, Remya V. M. (2004-11-25) hereby declare that this thesis entitled **'Biosurfactant producing bacteria from the selected soils of Kerala'** is a bonafide record of research work done by me during the course of research and this thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title of any other University or Society.

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Certified that this thesis, entitled **Biosurfactant producing bacteria from selected soils of Kerala'** is a record of research work done independently by **Ms. Remya V. M.** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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We, the undersigned members of the Advisory Committee of **Ms. Remya V.M.,** a candidate for the degree of **Master of Science in Agriculture** with major field in Plant Pathology, agree that the thesis entitled **"Biosurfactant producing bacteria from the selected soils of Kerala"** may be submitted by **Ms. Remya V. M.,** in partial fulfillment of the requirement for the degree.

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Remya. V.M.

(REMYA V M)

Dedicated to

my beloved

parents

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1. INTRODUCTION

Microorganisms produce a wide range of useful surface-active compounds called biosurfactants. These biomolecules act on the interfaces of two liquids and alter their physical conditions. Microbial surfactants or biosurfacants (BS) are important biotechnological products with a wide range of applications. Their properties of interest are (i) in changing surface and interfacial tensions (ii) wetting and penetrating action (iii) spreading (iv) hydrophilicity and hydrophobicity actions (v) microbial growth enhancement and (vi) antimicrobial actions (Kosaric, 2001). These biogenic surfactants can increase the surface area of hydrophobic pollutants like petroleum hydrocarbons, oil residues, pesticides and synthetic surfactants present in soil and water environment, thereby increasing their water solubility (Karanth *et al.,* 1999). Nowadays, biosurfactants have great demand in agriculture and industries.

Pollution caused by man-made, non-biodegradable organic chemicals, widely used in agriculture and industry has become a key issue of environmental safety. Accumulation and persistence of toxic materials in farm soils, irrigation and drinking water has become a threat today. Among the recalcitrants, aromatics and their chlorinated derivatives, pesticides like hexachlorophenols, chlorobenzenes, DDT, 2,4-D and dieldrin are largely non - biodegrable. They also bioaccumulated in the food chain. A number of physio chemical remediation processes have been employed from time to time, but these are expensive and inadequate for large scale application and do not successfully degrade many wastes (Kocher and Kahlon, 2003). Nowadays, surfactants of microbial origin are mainly used in handling industrial emulsions, control of oil spills, biodegradation of industrial effluents and contaminated soils.

There is immense scope for biosurfactants in the field of agriculture. Nowadays, synthetic additives are mainly used in pesticides as wetting, dispersing and suspending agents. Such synthetic substances can be replaced by microbial

surfactants as they do not contain any hazardous compounds. Similar applications are possible while manufacturing fertilizers, as the biosurfactant can prevent caking during storage and also help in the uniform dispersal of the applied fertilizers.

Uncontrolled and catastrophic release of pesticides poses ecological and environmental repercussions, as many of them are toxic and persistent in terrestrial and aquatic environments. Microbial degradation of pesticides had gained momentum in recent times. Several bacteria belonging to the genera, *Pseudomonas, Flavobacterium, Acinetobacter, Rhodococcus, Bacillus, Arthrobacter* and *Mycobacterium* are known to be efficient pesticide degraders. Those bacteria, which thrive in heavily hydrocarbon-loaded and toxic-polluted areas, would be of great value for the bioremediation of pollutants and pesticide contaminated soils, there by adding to human welfare.

In the light of the above facts, the study entitled "Biosurfactant producing bacteria from the selected soils of Kerala" was taken up with the following objectives.

1) Isolation and screening of biosurfactant producing bacteria from selected soil samples
2) Characterization of promising biosurfactant producing bacterial isolates
3) Estimation of biosurfactant production and bioactivity studies as evidenced by surface tension and emulsifying properties
4) Effect of nutritional and cultural conditions on the biosurfactant production and emulsification activity of the bacterial isolates
5) Studies on the degradation of pesticides by BS bacteria
6) Antimicrobial activity of biosurfactant bacterial isolates against soil borne pathogens and biocontrol agents
7) Plant growth promoting effect of selected biosurfactant producing bacteria

2. REVIEW OF LITERATURE

Biosurfactants or microbial surfactants are surface-active biomolecules produced by a variety of microorganisms. The enormous market demand for surfactants is currently met by numerous synthetics, mainly petroleum-based chemical surfactants. These compounds are usually toxic to the environment and non-biodegradable (Juwarker *et al.*, 1993). The production process and byproducts of synthetic surfactants may be environmentally hazardous. Strict environmental regulations and increase in awareness to protect the ecosystem have resulted interest in biosurfactants as alternative to chemical surfactants (Banat *et al.*, 2000).

Biosurfactants (BS) is beginning to acquire a status as potential performance - effective molecules in various fields. At present, BS is mainly used in studies on enhanced oil recovery and hydrocarbon bioremediation. Biosurfactants have potential applications in agriculture. Use of biosurfactants for degradation of pesticides in soil and water environment has also gained attraction nowadays. Surfactants can increase the surface area of hydrophobic materials, such as pesticides in soil and water environment, thereby increasing their water solubility. Hence the presence of surfactants may increase microbial degradation of pollutants (Karanth *et al.*, 1999).

Biosurfactants are used in various ways in the farm sector. They can be used along with phosphate fertilizers, as spray applications and in biological control. In phosphate fertilizers, biosurfactants are effective to prevent caking during storage. Mukherjee *et al.* (2006) described some practical approaches that have been adopted to make the BS production process economically attractive. These include the use of cheaper raw materials, optimized and efficient bioprocesses and overproducing mutant and recombinant strains for obtaining maximum productivity. The application of these strategies in biosurfactant production process leads to the successful commercial production of valuable and versatile biomolecules in the near future.

2.1. ISOLATION OF BIOSURFACTANT PRODUCING BACTERIA FROM DIFFERENT ECOSYSTEMS

Biosurfactants (BS) is surfactants produced by some microorganisms extra cellularly or as an ingredient of cell membrane. Suzuki *et al.* (1969) isolated BS with strong emulsifying properties from *Arthrobacter paraffinens* grown on liquid paraffins. BS from bacteria belonging to the genus *Arthrobacter, Mycobacterium, Brevibacterium, Corynebacterium* and *Nocardia* were isolated from hydrocarbon-contaminated sites, and were characterized as trehalose lipids (Suzuki *et al.*, 1969; Duvnjak *et al.*, 1982; Banerjee *et al.*, 1983). Sethunathan and Yoshida (1973a) isolated a *Flavobacterium* sp. from diazinon treated rice fields that hydrolysed diazinon.

Singer and Finnerty (1990) isolated BS producing *Rhodococcus* sp. from oil enriched soil following several passages on hexadecane. Ruwaida *et al.* (1991a) reported a gram positive, non-fermentative, rod shaped bacterium identified as *Rhodococcus* isolated from Kuwait soil, grown on hydrocarbons such as kerosene and n-paraffin as substrates. Rocha *et al.* (1992) isolated two strains of BS bacteria, identified as *Pseudomonas aeruginosa* from injection water and crude oil associated water in Venezuelan oil fields and the BS produced stable emulsions of heavy and extra - heavy crude oils, reducing the surface tension of water from 72 to 28 dynes cm^{-1}. Hwang (1993) reported that the bacterium *Klebsiella oxytoca* isolated from oil contaminated soil released extra cellular biosurfactant when it was cultured on water insoluble aliphatic compounds as a carbon source.

Iqbal *et al.* (1995) isolated a gamma - ray induced mutant of *Pseudomonas aeruginosa* str. S8. Its mutant (EBN - 8) produced more biosurfactants and showed three to four times more hydrocarbon emulsification activity when grown on Khaskheli crude oil with hexadecane as carbon and energy source. Yakimov *et al.*

(1995) reported that a bacterial isolate *Bacillus licheniformis* str. BAS 50, from a petroleum reservoir, produced a lipopeptide surfactant lichenysin A, when cultured on substrates with salinities 13 per cent NaCl. Arino *et al.* (1996) isolated a glycolipid producing bacterium, *Pseudomonas aeruginosa* GL1 from the soils of gas manufacturing plant contaminated with polycyclic aromatic hydrocarbons (PAH). Mallik *et al.* (1999) isolated *Arthrobacter* sp. from methyl parathion contaminated soil that degrades chlorpyriphos effectively. Balasankar and Nagarajan (2000) isolated a fast growing *Bacillus brevis* from coal carbonized waste water that effectively utilized phenols as the carbon and energy source.

Bhadbhade *et al.* (2002) isolated two cultures viz., *Arthrobacter atrocyaneus* MCM B - 425 and *Bacillus megaterium* MCM B - 423, by selective enrichment and adaptation culture technique from soil exposed to pesticide, monocrotophos. Yateem *et al.* (2002) isolated and characterized two BS producing *Pseudomonas aeruginosa* strains (KISR C1 and KISR B1) from Kuwaiti oil contaminated soil which differed in their biosurfactant stimulating carbon source, nitrogen concentration and the pH of the medium. Among this, B1 strain was found to be very effective in the emulsification of crude oil. Bodour *et al.* (2003) reported that BS producing microorganisms were found in most soils and their distribution was dependent on soil conditions. Gram positive BS isolates tend to be from heavy metal contaminated soils and gram negative isolates tend to be from hydrocarbon contaminated soils.

Syal and Ramamurthy (2003) isolated hydrocarbon utilizing bacteria belonging to the genus *Acinetobacter* from diesel and kerosene contaminated soil samples, potentially contributed to the remediation of hydrocarbon spills, particularly aliphatic compounds due to its capacity to produce surface active agents. Rahman *et al.* (2003) studied the distribution of biosurfactant producing and crude oil degrading bacteria from oil contaminated environments. They isolated 32 oil-degrading bacteria from 10 different oil contaminated sites of gasoline and diesel fuel

stations. Among this, 80 per cent exhibited biosurfactant production which included *Pseudomonas* spp., *Micrococcus* spp., *Bacillus* spp., *Corynebacterium* spp., *Flavobacterium* spp. and *Acinetobacter* spp., which emulsified xylene, benzene, n-hexane, Bombay High crude oil, kerosene, gasoline, diesel fuel and olive oil.

Perusal of current literature on degradation of xenobiotics indicates that bacteria are the major degraders of persistent pollutants. Kocher and Kahlon (2003) isolated and characterized a number of *Pseudomonas* strains that could degrade organochlorinated pesticides and chlorobenzoates. Singh *et al.* (2004) isolated a chlorpyriphos degrading bacteria *Enterobacter* strain B-14 from an Australian soil and the strain had the ability to utilize chlorpyriphos as the sole source of carbon and phosphorus. Kuiper *et al.* (2004) isolated *Pseudomonas putida* strain PCL 1445 from roots of plants grown on a site polluted with polycyclic aromatic hydrocarbons and found to produce lipopeptide biosurfactant Putisolvin I and Putisolvin II with surface tension reducing activity. Dubey and Juwarker (2004) reported that *Pseudomonas aeruginosa* strain BS2 isolated from distillery and whey wastes has ability to produce potent biosurfactant, an eco-friendly substitute for synthetic surfactants and also capable of reducing the pollution load of these wastes in the range of 85-90 per cent.

Zhang *et al.* (2004) isolated a novel bacterium *Paracoccus*, from Greek soils contaminated with polycyclic aromatic hydrocarbons capable of degrading PAH and opined that it may be useful for bioremediation. Christova *et al.* (2004) studied the enhanced hydrocarbon biodegradation and rhamnolipid biosurfactant production of a newly isolated *Bacillus subtilis* strain. 22 BN. Benincasa *et al.* (2004) isolated *P. aeruginosa* LB1 from petroleum contaminated soil which produced rhamnolipids (RLLB1) when cultivated on soap stock as the sole carbon source. Hasanuzzaman *et al.* (2004) isolated a novel oil degrading bacterium identified as *P. aeruginosa* T1 from a hot spring in Hokkaido, Japan. This strain secreted a fatty acid inducible extra

cellular lipase that efficiently degraded different types of fats and oils including edible oil waste.

Joshu *et al.* (2005) isolated a novel bacterium *Pseudoxanthomonas kaohsiungensis* sp. nov., from oil polluted sites in Southern Taiwan. The culture supernatant of the strain reduced the surface tension of the medium from 68 to 32.6 dyne cm^{-1}, when olive oil used as sole carbon and energy source. Penta chlorophenol (PCP) is one of the major industrial pollutants from the industries such as pesticides, fertilizers and oil refineries. Nandish and Jagadeesh (2006) isolated *Enterobacter* NV-5 after selective enrichment of soil samples collected from different industrial dump sites and found that the strain degraded PCP by 72 per cent in five days under optimum pH condition. Tonkova *et al.* (2006) isolated *P. fluorescens* strain HW-6 from industrial wastewater that produced glycolipid BS at high concentrations 1.4-2.0 g l^{-1}, when grown on hexadecane as a sole carbon source.

2.2. IMPORTANT BIOSURFACTANT PRODUCING BACTERIA AND NATURE OF BIOSURFACTANT PRODUCTION

Biosurfactants or microbial surfactants are broadly classified into glycolipids, surface-active antibiotics, polymeric microbial surfactants and particulate surfactants. Glycolipids are the most common types of biosurfactants. Glycolipids can be categorized as trehalose lipids, sophorolipids and rhamnolipids. One of the best studied glycolipids are the rhamnolipid, produced by several species of *Pseudomonas* (Hauser and Karnovsky, 1954). Suzuki *et al.* (1969) reported *Arthrobacter paraffinens* produced Trehalolipids in the emulsion layer of culture broth when the cells were grown on hydrocarbon substrates.

The most commonly reported genera of hydrocarbon - degraders comes under the genera *Pseudomonas, Acinetobacter, Nocardia, Vibrio* and *Achromobacter*

(Floodgate, 1984). The surface-active agents that facilitate microbes to degrade hydrocarbons are increasingly popular due to diversity in their activity and for their applicability (Rosenberg, 1986; Desai and Banat, 1997). *Acinetobacter calcoaceticus* A_2 produced an extra cellular anionic polysaccharide surfactant of molecular mass 51.4 Kda that dispersed limestone and titanium dioxide (Rosenberg *et al.*, 1988). The biopolymer referred to as biodispersan, binds to powdered calcium carbonate and changes its surface properties in such a way that it helped better dispersion of water. In *Rhodococcus erythropolis*, glycolipids were found as trehalose dimycolates (Kim *et al.*, 1990).

In an oil displacement assay Morikawa *et al.* (1993) studied a lipopeptide biosurfactant termed as 'arthrobactin' produced by *Arthrobacter* sp. strain MIS 38 removed oil more effectively than synthetic surfactants, such as Triton X - 100 and sodium dodecylsulphate. *Bacillus licheniformis* JF-2 produced a very active BS lichenysin, a lipopeptide with a molecular weight of 1,035. This BS was very similar to that of surfactin, a lipopeptide produced by *Bacillus subtilis*. Under optimal conditions, *B. licheniformis* JF-2 reduced the interfacial tension against decade to $6x10^{-3}$ dyne cm^{-1}, which is one of the lowest interfacial tensions ever reported for a microbial surfactant (Lin *et al.*, 1994). *Pseudomonas maltophilia* CSV 89, a soil bacterium, produced an extra cellular BS 'Biosur-Pm', a partially purified product composed of 50 per cent protein and 12-15 per cent sugar, helped in the assimilation of hydrocarbon (Phale *et al.*, 1995).

Venezia *et al.* (1995) reported bioemulsifiers of *Acinetobacter*. The first well studied *Acinetobacter* bioemulsifier is RAG - 1 emulsion, a complex of an anionic heteropolysaccharide and protein where as, Alasan another one produced by a strain of *Acinetobacter radioresistens,* a complex of an anionic polysaccharide and protein with a molecular weight of approximately $1x10^6$. *Acinetobacter* sp. produced a group of high molecular weight biosurfactants known as bioemulsions (Rosenberg and Ron,

1998). The biosurfactant surfactin obtained from *Bacillus subtilis* was highly effective for the removal of heavy metals from contaminated soils and sediments and has been reported to be the most active biosufactant that has been discovered to date (Cooper *et al.*, 1989; Mulligan *et al.*, 1999). Kosaric (2001) reported in the presence of selected biosurfactants a preferential and significant removal of polyaromatic hydrocarbons (PAHs) was observed after 22 days of bioremedition.

Several studies showed that *Pseudomonas aeruginosa* isolated from different hydrocarbon contaminated sites produced extra cellular glycolipids called rhamnolipids, with surface-active properties (Arino *et al.* 1996; Deziel *et al.* 2003; Benincasa *et al.* 2004). Kuiper *et al.* (2004) isolated *Pseudomonas putida* strain PCL 1445 from roots of plants grown on a site polluted with polycyclic aromatic hydrocarbons, produced lipopeptide biosurfactant Putisolvin I and Putisolvin II, which showed surface tension reducing activity. Gunther *et al.* (2005) reported that *Pseudomonas chlororaphis*, a non-pathogenic saprophyte of the soil, produced later rhamnolipid biosurfactant.

2.3. SCREENING OF BIOSURFACTANT BACTERIA AND ESTIMATION OF BIOSURFACTANT PRODUCTION

Although various BS possess different structures, there are some general phenomena concerning their biosynthesis. For example, biosurfactant production can be induced by hydrocarbons or other water insoluble substrates (Reisfeld *et al.*, 1972). Kiyohara *et al.* (1982) devised a method to screen BS bacteria that degraded solid hydrocarbons such as the polycyclic aromatic hydrocarbons phenanthrene, anthracene and biphenyl. They also identified certain phenanthrene assimilating bacteria including *Alkaligenes faecalis* AFK 2, *Beijerinckia* Bwt, and *Pseudomonas* SPM 64, which gave clear zone on phenanthrene covered agar plate. This method

was also applicable for the detection of bacteria able to assimilate anthracene, naphthalene and biphenyl.

Duvnjak and Kosaric (1985) reported that *Corynbacterium lepus* produced large amount of biosurfactants, but they remained cell bound. The surfactant was released from cells only when it was treated with hexadecane. This suggests that hydrocarbons are necessary for the release of biosurfactants from the culture broth.

A rapid and sensitive method was devised by Jain *et al.* (1991) to screen bacterial colonies that produced surfactants. Drops of cell suspensions of surfactant producing colonies collapsed on oil-coated surface and those, which did not produce or produced very low concentration of surfactants remained stable. The stability of drops was dependent on biosurfactant concentration and it correlated with surface tension but not with emulsifying activity. Microbial colonies grown on hydrocarbons could readily be screened for surfactant production by this method. Siegmund and Wagner (1991) reported agar plate method for rapid screening of biosurfactants. Bacteria were streaked on plates containing mineral salt agar medium. At 24 h of incubation, presence of dark blue haloes surrounding the colonies indicated the presence of BS, rhamnolipid.

The choice of method for recovery of a particular biosurfactant depends on its ionic charge, solubility in water, whether the product is cell bound or extra cellular and of course, the cost of recovery. The methods generally used for biosurfactant recovery include solvent extraction, adsorption followed by precipitation, crystallization, centrifugation and foam fractionation. Most biosurfactants were secreted into the medium and thus isolated from either culture filtrate or supernatent obtained after removal of cells (Shafi and Khanna, 1995).

Burd and Ward (1996) screened polycyclic aromatic hydrocarbon (PAH) degrading bacteria *Pseudomonas marginalis* PD–14B by phenanthrene spray method on the pre incubated bacterial colonies on nutrient agar plates. The colonies were generated a clear zone against the opaque background of the PAH, due to the solubilization of these compounds mediated by biosurfactants released by the cells into the agar zone surrounding the colony. Some findings showed that microbial BS, lipase production can be induced by the presence of lipids such as Triacyl glycerol (TG) and free fatty acid (FFA), and by the presence of cyclohexane in the medium (Ogino *et al.*, 1999).

Perusal of literature indicated various ways for the estimation of biosurfactant production. Syal and Ramamurthy (2003) studied the ability of a bacterial isolate D_2 (*Acinetobacter* sp.) from diesel and kerosene contaminated soils to produce surfactant in liquid culture, by supplementing different carbon sources (1%) in to Bushell - Hass medium. Deziel *et al.* (2003) reported the biosurfactant production and analysis of *Pseudomonas aeruginosa* by growing them in 50 ml iron-limited mineral salt medium supplemented with 20 per cent mannitol. Surface and wetting activities were qualitatively compared with the drop collapsing test and surface tension was measured by the ring method with a due Nouy tensiometer.

Hasanuzzaman *et al.* (2004) studied visible lipolytic activity of *P. aeruginosa* strain T_1 by forming a clearance halo around the bacterial colony after growth for 3 days on an agar plate made turbid with emulsified salad oil, indicated the presence of an extra cellular lipase. Youssef *et al.* (2004) reported the use of drop collapse method as a primary method to detect biosurfactant producers, followed by the determination of the biosurfactant concentration using the oil spreading technique and suggested this as a quick and easy protocol to screen and quantify BS production. Gunther *et al.* (2005) purified rhamnolipids from *Pseudomonas chlororaphis* by separating the cells from the supernatant by centrifugation.

2.4. FACTORS AFFECTING THE RATE OF BIOSURFACTANT PRODUCTION AND BIOACTIVITY STUDIES

The yield of biosurfactants greatly depends on the nutritional and environmental conditins of the growing organism (Santos *et al.* 1984; Karanth *et al.* 1999; Salleh *et al.*2003). Shafeeq *et al.* (1991) compared the BS production of six *Pseudomonas* strains by growing them on n-hexadecane. Supernatant from whole culture broth of these strains lowered the surface tension (ST) from 65 m Nm^{-1} to 1-3 m Nm^{-1} and interfacial tension from 40 m Nm^{-1} to 1-3 m Nm^{-1}, biosurfactant property retained in the culture broth up to 80^0C, at pH13 and at sodium chloride concentration of 17 per cent. This indicated their possible role in some depleted oil wells.

Ruwaida *et al.* (1991b) reported the nutritional requirements and growth characteristics of a BS producing *Rhodococcus* bacterium isolated from Kuwait soil. The bacterium was grown on hydrocarbon as the sole carbon source. Maximum cell yield and BS production were obtained when medium includes two per cent n-paraffin as carbon and energy source, or 0.2 per cent lactose broth, or optimum concentration of nitrogen, phosphorus, iron, magnesium and sodium sources and small concentration of potassium as true element sources. Rocha *et al.* (1992) reported that two strains of BS producing *P. aeruginosa* from injection water and crude oil associated water produced BS and reduced the ST of water from 72 to 28 dynes cm^{-1}. pH, temperature, salinity or Ca^{2+} or Mg^{2+} concentrations did not affect tensio-actve properties of BS.

Marcin *et al.* (1993) reported that olive oil induced BS lipase production in *P.aeruginosa* MB5001. Banat (1993) isolated a thermophilic *Bacillus* strain on a hydrocarbon containing medium grown up to 50 ^{0}C and produced BS that emulsified kerosene and other hydrocarbons efficiently. Hwang (1993) reported that the

bacterium *Klebsiella oxytoca* produced significant amount of extra cellular BS when grown on a medium containing inorganic nitrogen and water soluble carbohydrate as carbon source. Maximum emulsifying activity was obtained when the bacterium was cultured under optimum conditions (pH 7.0, and temperature 30 ^{0}C).

Hommel and Huse (1993) found that *Torulopsis apicola* IMET 43747 produced large amount of BS sophorolipids during the stationary growth phase on glucose, fructose or sucrose, but the amount of biosurfactant was negligible when it was grown on maltose or galactose. Mercade *et al.* (1993) studied olive oil mill effluent (OOME) used as new substrate for BS production. Among the different BS producing strains, several strains of *Pseudomonas* sp. were able to grow on OOME as the sole carbon source. Chavez and Palmeros (1994) observed that the genus *Pseudomonas* secreted extra cellular BS lipases on medium, which were found to be thermo resistant and very active at alkaline pH. The bacterium was capable of growing in temperature ranges between 15^{0}C and 55^{0}C. Mercade and Manresa (1994) reported that urban wastes, peat pressate and agro industrial byproducts such as OOME can be used as new substrates for microbial growth for the production of biosurfactant.

Phale *et al.* (1995) found that *Pseudomonas maltophilia* when grown at a pH 8.0 produced high amount of BS 'Biosur-Pm' and showed higher affinity for hydrocarbons compared to the cells grown at pH 7.0. Deziel *et al.* (1996) suggested a method for the detection of biosurfactant production by ST lowering and the emulsification activity of PAH utilizing bacteria grown on iron limited salt medium supplemented with high concentrations of dextrose or phenanthrene. The bacterium *P. aeruginosa* 195 J gave maximum BS on naphthalene compared with that on mannitol. Arino *et al.* (1996) found that *P.aeruginosa* GL 1 stimulated rhamnolipid production in nitrogen limited conditions by using glycerol, yielded higher production of rhamnolipid than the other hydrophobic carbon sources.

Makkar and Cameotra (1997) reported that two strains of *Bacillus subtilis* MTCC 2423 and MTCC 1427 produced BS when grown on medium containing two per cent total sugars as cane molasses. The BS reduced the ST of the medium to 29 and 31 dynes cm^{-1}. This was the first report of BS production by strains of *B. subtilis* at 45^{0}C. Makkar and Cameotra (1998) examined the factors influencing *B. subtilis* for BS production, by growing them on different carbon and nitrogen sources with varying temperature like 45 ^{0}C and 30 ^{0}C. At 45 ^{0}C the ST of cell - free broth decreased to 34 dynes cm^{-1}on two per cent sucrose after 72h, or to 30-32 dynes cm^{-1}on starch after 24-96 h. At 30 ^{0}C more BS was formed and lowered the ST to 27 - 29dynes cm^{-1}. The BS was stable at 100^{0}C and within a wide pH range (3-11). Lang *et al.* (1998) described about BS bacteria *Rhodococci,* produced glycolipid BS molecule in presence of alkanes with ST lowering property. Lang and Wulbrandt (1999) found that the individual rhamnolipids of *Pseudomonas* sp. in soyabean oil as sole source were able to lower the ST of water from 72 m Nm to 25 - 30 m Nm at concentrations of 10-200 mg l^{-1}

There are some reports on microbial lipase production induced by the presence of lipids like triacylglycerol and free fattyacid, and by the presence of cyclohexane in the medium (Ogino *et al.* 1999 ; Dharmasthiti and Luchai, 1999; Ito *et al.* 2001). The BS produced by *Pseudomonas* sp. by emulsified kerosene and vegetable oil, that lowered the ST of water to 25 mNm^{-1} at a pH of 10.5 when BS were taken at a concentration of 0.5 g l^{-1}. (Vipulanandan and Ren, 2000).

According to Ito *et al.* (2001) organic solvent–tolerant *P. aeruginosa* LST - 03 produced extra cellular lipase when grown in a synthetic medium containing some lipids as the sole carbon source. Syal and Ramamurthy (2003) studied that the isolate *Acinetobacter* produced BS in liquid culture supplemented with different carbon sources and the isolate was grown rapidly and profusely in glucose, but poorly in sucrose because it was unable to metabolize sucrose rapidly. In case of hydrocarbons,

significant amount of surfactant activity was observed compared to other sugar sources.

Hasanuzzaman *et al.* (2004) studied *P. aeruginosa* T1 induced lipase by culturing them in mineral salt medium supplementing 0.5 per cent glucose and salad oil stimulated lipase activity along with increased biomass production. Shin *et al.* (2004) described the solubilization and degradation of phenanthrene at different pH by the addition of rhamnolipid BS solution. Tonkova *et al.* (2006) reported that the glycolipid BS produced by *P. fluorescens* HW - 6 decreased the ST of the air or water interface by 35 m Nm. and efficiently emulsified aromatic hydrocarbons, kerosene, n-paraffins and mineral oils.

2.5. ROLE OF BACTERIA ON DEGRADATION OF PESTICIDES

Pesticide usage in India is vastly different from that in many developed countries. Unscientific and indiscriminative use of pesticides by the farmers has aggravated the problems caused by pesticides. In order to safeguard the environment and public health, degradation of residual pesticide is essential. It may be generalized that pesticide contamination problems in the environment are directly related to their persistent nature, the most important factor determining the degree of persistence being the chemical characteristics of the pesticide compound itself. The most important degradation force operating on chemicals in the environment are microorganisms. They can degrade a wide variety of pesticides and their residues

According to Stanier (1947), microbes are known to metabolize a homologous series of compounds once adapted to metabolize a single member of the series. Miyamoto *et al.* (1966) studied that *B. subtilis* converted fenitrothion to corresponding amino analogs by reductive process on the pesticidal chemical. Sethunathan and Yoshida (1973a) isolated *Flavobacterium* sp. from diazinon-treated

rice field soils that readily hydrolysed diazinon and mineralized the pyrimidinyl moiety to carbon dioxide in a mineral salt medium supplemented with the insecticide.

Two bacterial strains, *Flavobacterium* sp.strain ATCC 27551 and P*seudomonas diminuta* strain GM were isolated by Sethunathan and Yoshida (1973b), as organophosphate degrading bacteria from different soils in the Phillippines and United States respectively. Kaufman (1974) reported that Dithane M-45, nabam, maneb, zineb and polyram yield ethylene thiourea (ETU) during biodegradation and got readily decomposed in soil to ethylene urea and then to carbon dioxide and other metabolites.

Several investigations suggested that the formulation of pesticides affected its degradability. Davis and Kuhr (1976) examined that decomposition of granular chlorpyriphos was slower than that of the emulsifiable concentrate. Furukawa *et al.* (1978) found that *Alkaligenes* and *Acinetobacter* strains actively degraded penta chloro benzene isomers. Balasubramanya *et al.* (1980) reported that a strain of *Pseudomonas* sp. converted carboxin to in the order of sulfoxide, sulfone, 2-(Vinyl sulfonyl) acetanilide, 2-(2-hydroxy ethylsulphonyl) acetic acid, aminophenol, ammonium and nitrite.

Mancozeb a member of ethylene bisdithiocarbamate (EBDC) fungicides, has a negligible vapor pressure, and low potential to volatilize into the air. The mineralization of mancozeb degradation to carbon dioxide was mainly done by the microorganisms (Lyman and Lacoste 1975; Ligocki and Pankow, 1989). Studies of Rani and Kumari (1994) revealed that a strain of *Pseudomonas putida* hydrolyzed methyl parathion using p-nitrophenol as a sole source of carbon.

Mallik *et al.* (1999) reported that an *Arthrobacter* sp. isolated from methyl parathion enriched soil degraded chlorpyriphos in mineral salt medium. Girija *et al.*

(2000) studied on degradation of lindane activity using non-fluorescent *Pseudomonas* sp. isolated from sugarcane fields of Kerala. Chlorpyriphos was found to be degraded co-metabolically in liquid media by *Flavobacterium* sp. and also by *Escherichia coli* clone with organophosphorus degrading (*opd*) gene (Wang *et al.* 2002).

Perusal of literature showed that a diverse group of bacteria including the members of the genera *Alcaligenes, Flavobacterium*, *Pseudomonas* and *Rhodococcus* metabolized pesticides (Aislabie and Jones, 1995; Gowrisankar *et al.*, 2002). Bhadbhade *et al.* (2002) isolated two cultures viz., *Arthrobacter atrocyaneus* MCM B-425 and *Bacillus megaterium* MCM B-423, from soil exposed to monocrotophos and reported to be degraded monocrotophos to carbon dioxide, ammonia and phosphates through formation of one unknown compound metabolite 1, valeric or acetic acid and methyl amine as intermediate metabolites. Awasthi *et al.* (2003) isolated an efficient strain, *Bacillus subtilis* MTCC 1427 which produced BS, surfactin capable of degrading endosulfan. Singh *et al.* (2004) isolated a chlorpyriphos degrading bacteria *Enterobacter* strain B - 14 from Australian soil and the strain had the ability to utilize chlorpyriphos as the sole source of carbon and phosphorus. The isolate hydrolysed chlorpyriphos to diethyl thiophosphate (DETP) and 3, 5, 6-trichloro–2- pyridinol. Vancov *et al.* (2005) developed an encapsulated procedure for the atrazine degrading bacteria *Rhodococcus erythropolis* N186/21, for slow release of *R. erythropolis* and found that encapsulated cells of *R. erythropolis* effectively reduced atrazine residues.

2.6. ANTIMICROBIAL ACTIVITY OF BIOSURFACTANT PRODUCING BACTERIA

The antagonistic property of the biosurfactant bacteria to ward off plant pathogens has been reported by several authors. Antiviral activity of the biosurfactant viscosin produced by *Pseudomonas viscosa* and *P. fluorescens* was reported by

Groupe *et al.* (1951). Certain biosurfactants, mainly lipopeptides and glycolipids have antibiotic and / or biostatic properties (Kurioka and Liu, 1967; Bernheimer and Avigad, 1970). Asaka and Shoda (1996) reported that *Bacillus subtilis* RB 14 which produced surfactin, and solubilized the cell envelope components of several plant pathogens and thus it suppressed their growth. Fluorescent *Pseudomonas* sp. are known to inhibit plant pathogenic fungi in sugarbeet rhizosphere and members of both the *Pseudomonas fluorescens* - *Pseudomonas putida* species complex and *P. chloraphis* (including *P. aureofaciens*) have demonstrated *in vitro* antagonism towards several soil micro fungi, but with great variability among the strains (Nielsen *et al.*1998).

Nielsen *et al.* (1999) reported that *P. fluorescens* DR 54 showed antagonistic properties against plant pathogenic *Pythium ultimum* and *Rhizoctonia solani* both *in vitro* and *in planta*. The antifungal compound cyclic lipodepsipeptide, viscosinamide extracted from this bacterium also exhibited strong biosurfactant activity. *In vitro* tests showed that the purified viscosinamide reduced fungal growth and aerial mycelium development of both *P. ultimum* and *R. solani*.

Bacillus brevis acts as biocontrol agent and it has an antifungal metabolite gramicidin S and a biosurfactant, which acts directly against *Botrytis cinerea* conidial germination and to some extent mycelial growth. Gramicidin S and *B. brevis* inhibited *Sphaerotheca fuliginea* both *in vitro* and *in vivo* (Hellen *et al.* 1996; Seddon and Schimitt, 1999). Nielsen *et al.* (2002) reported that fluorescent *Pseudomonas* sp. produced cyclic lipopeptides (CLPs), which exhibited strong biosurfactant properties and also had antibiotic properties towards root-pathogenic microfungi.

Biosurfactants are produced by a variety of microorganisms and have been shown to be involved in bioremediation of xenobiotics and biological control of plant pathogens. Several strains of *Pseudomonas* species present in the rhizosphere soil of

plants are capable of producing biosurfactants and have received more attention during recent years as potential biocontrol agents of plant pathogenic Oomycetes (*Phytophthora* sp.) in different crops. Nielsen and Sorensen (2003) studied the *Pseudomonas fluorescens* strains DR 54, 96.578 and DSS 73 produced three different cyclic lipopeptides viscosinamide, tensin and amphisin. All showed antagonistic activities against the important plant - pathogenic microfungi *P. ultimum* and *R. solani*.

Pseudomonas aeruginosa that produced rhamnolipid biosurfactant also exhibited anti - phytopathogenic action (Mulligan and Gibbs, 2004). Benincasa *et al.* (2004) studied that the isolate *P. aeruginosa* LBI showed anti-microbial activity against *Alternaria* sp., *Pencillium* sp. and *Chaetomium globosum*. Gunther *et al.* (2005) reported that *Pseudomonas chlororaphis* a non-pathogenic saprophyte of the soil, which produced rhamnolipid biosurfactant, and used as a biocontrol agent against certain phytopathogens. Jonghe *et al.* (2005) reported that rhamnolipid, extra cellular metabolites of *P. aeruginosa* with surfactant properties was very effective in controlling the spread of brown root rot disease caused by *Phytophthora cryptogea* in the hydroponic forcing system of witloof chicory, and the rhamnolipid BS can be used as a protectant against brown root rot disease.

3. MATERIALS AND METHODS

The present study entitled "Biosurfactant producing bacteria from the selected soils of Kerala" was conducted at the Department of Plant Pathology, College of Horticulture, Vellanikkara. Experiments on degradation of pesticides by the biosurfactant (BS) producing bacteria were carried out at Radio Tracer Laboratory, College of Horticulture, Vellanikkara and also at Pesticide Residue Laboratory, College of Agriculture, Vellayani during the period 2004-2006.

The details of the materials and the techniques used for the investigation are described below.

3.1. COLLECTION OF SOIL SAMPLES

Different soils viz., forest soils, soils from automobile fuel stations and lubricant spill overs, soils from the drains of ayurvedic nursing homes, soils from the plots of permanent herbicidal and pesticidal trials were used for the present study. Details of the soil samples collected are presented in Table 1. A quantity of 500g each of the soil samples was collected from various locations, air dried and stored under laboratory conditions in polythene bags.

3.2. ENRICHMENT OF SOIL SAMPLES

Different soil samples of 100g each were taken in 250 ml conical flasks, and treated with 10 ml each of crude oil, neem oil and sterile water and incubated at room temperature (28 ± 2^0C) for 30 days. Samples were moistened with sterile water to avoid desiccation.

Table 1 Sources of hydrocarbon contaminated soil samples

Sl.No.	Code No.	Source of soil sample
1.	EFP-1	*Eucalyptus* Plantations, Kerala Forest Research Institute, Peechi
2.	FSW-2	Forest lands, Wyanad
3.	IOP-1	Indian Oil Petroleum Bunk, Peringottukara, Thrissur
4.	MSP-2	Maruti automobile service station, Pazuvil, Thrissur
5.	VAP-1	Vydyaratnam Ayurveda Pharmacy, Perumbilllisery , Thrissur
6.	KAC-2	Kunnathuvalappil Ayurveda Nursing Home, Cherpu, Thrissur
7.	HSM-1	Herbicide treated plot, Agricultural Research Station, Mannuthy (Location 1)
8.	HSM-2	Herbicide treated plot, Agricultural Research Station, Mannuthy (Location 2)
9.	CSM-1	Chlorpyriphos treated plot, Mannarkad, Palakkad
10.	MSV-1	Mancozeb treated vegetable plot, College of Horticulture, Vellanikkara

3.3.ISOLATION OF HETEROTROPHIC BACTERIA FROM SOIL SAMPLES

Total heterotrophic bacteria were isolated from the soil samples by adopting serial dilution plate technique (Johnson and Curl, 1972). Representative soil samples of 10 g each were taken and suspended in 90 ml sterile water in conical flasks to make 10^{-1}dilution (1:10). The flasks were shaken well on a rotary shaker for 20 min to obtain a uniform suspension of microorganisms and samples were serially diluted upto 10^{-7}dilution. From this, one ml of suspension were withdrawn and transferred into sterile Petri dishes.

In the meantime, selective medium (Appendix I) (Gilstrap *et al.,* 1983) for the total heterotrophic bacteria were prepared and sterilized. The media were cooled and poured on to Petri dishes and were rotated in clockwise and anti clockwise directions for even spreading and was allowed for solidification. Then plates were incubated at room temperature.

After 48h, bacterial colonies appeared on the plates and the number of colonies per plate was counted and the number of colony forming units (cfu) per gram of soil sample computed using the formula,

$$\text{cfu/g of soil} = \frac{\text{Mean no: of colonies / plate}}{\text{Weight of soil taken}} \times \text{Dilution factor}$$

Morphologically distinct colonies were then picked up, purified and maintained on nutrient agar (Appendix II) slants and were numbered.

3.4. DETECTION OF BIOSURFACTANT PRODUCING BACTERIA

Bacteria isolated from different soil samples were detected for the biosurfactant property using inorganic salt medium (Appendix III) (Baruah *et al.,* 1997). A loopful of bacterial isolate was inoculated into 5 ml ISM broth taken in a test tube and incubated at room temperature for 24 h. After incubation, five ml of culture broth was again inoculated in 100ml of ISM broth taken in conical flasks. The flasks were incubated for five days at room temperature in a rotary shaker adjusted to100 strokes per minute. After five days, the flasks were withdrawn and bacterial isolates were screened for biosurfactant production.

3.4.1. Screening of bacterial isolates for surfactant production activity

3.4.1.1. Drop collapse technique

The experiment was conducted based on the technique followed by Jain *et al.* (1991). Clean glass plates of 10 x 15 cm size were taken and cleaned with alcohol to make them grease free and allowed to dry. Then plates were coated with a thin film of neem oil. Using a micropipette 0.05ml ISM broth containing test bacterium was placed as a drop on the glass plate. 15 to 20 drops could be placed on each plate at a time. Suitable controls were also maintained with uninoculated broth. Observations were made after one minute for the biosurfactant activity. Presence of uncollapsed drops indicated the absence of biosurfactant activity, where as collapsed drops proved that the particular bacteria produced biosurfactants. The extent of collapse was worked out by measuring the area of dispersion using a millimeter graph sheet, and thus the biosurfactant activity of the bacterial isolates was assessed.

3.4.1.2. Xylene spray method

Burd and Ward (1996) suggested another method for the detection of biosurfactant producing bacteria. The bacterial isolates were inoculated on nutrient agar plates and incubated at room temperature for 24 h followed by spraying with xylene. Immediately, a clear zone was formed around each bacterial colony due to the BS production.

3.4.2. Selection of biosurfactant producing bacteria

The bacterial isolates that showed maximum area of dispersion in drop collapse test and maximum area of clear zone in xylene spray method were selected for further studies.

3.4.ESTIMATION OF BIOSURFACTANT PRODUCING BACTERIAL POPULATION IN SELECTED SOIL SAMPLES

Among the heterotrophic bacterial isolates obtained from each soil samples, number of BS producing isolates were worked out from the screening tests. From this per cent BS producing bacterial isolates presented in the soil samples was estimated.

3.5.CHARACTERISATION OF BIOSURFACTANT PRODUCING BACTERIAL ISOLATES

Eight bacterial isolates that produced biosurfactant were characterized based on the cultural morphological and biochemical characters. Among the eight isolates, the promising isolates viz., MCN-3 and PFC-4 were sent to Microbial Type Culture Collections (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh for identification.

3.6.1. Cultural characters

3.6.1.1. *Colony characteristics of bacterial isolates*

Colony characteristics of the eight selected bacterial isolates were studied on yeast extract glucose agar (YEGA) medium (Appendix IV) (Gilstrap *et al.,* 1983). The bacterial isolates were streaked on the medium poured in Petri plates and incubated at room temperature. The shape, margin, colour, elevation and surface of bacterial colonies were examined 24h after incubation.

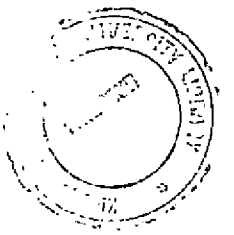
3.6.2. Morphological characters

3.6.2.1. Gram staining

Hucker's modification of Gram staining was done (Hucker and Conn,1923). A smear of each bacterial isolate was prepared on a clean glass slide and heat fixed over a flame by gentle intermittent heating. It was stained with Hucker's ammonium crystal violet solution (Appendix V) for one minute and then washed in a gentle stream of running tap water. After washing, it was flooded with Gram's iodine solution (Appendix V) for one minute and then decolourised with 95 per cent ethanol. After washing again in a stream of running tap water, the slide was stained with safranin solution (Appendix V) for one minute and the excess stain was washed off in tap water. After drying between folds of filter paper, the slide was examined under microscope for Gram reaction.

3.6.2.2. Spore staining

The smear of bacterial isolates were prepared and fixed on a clean glass slide. It was stained with malachite green solution (Appendix V) and allowed to react in the cold for 30 to 60 seconds and then showed under flame for 30 seconds. The smear was rinsed with water and stained with aqueous solution of safranin for 30 seconds. The smear was then rinsed with water, blotted dry and observed under oil immersion objective of the microscope.



3.6.3. Biochemical characters

3.6.3.1. Potassium hydroxide test

A loopful each of the bacterial isolate was put on a clean glass slide. One drop of three per cent potassium hydroxide solution was placed over it and thoroughly mixed with the help of a needle. Formation of highly viscous thin threads indicated Gram negative bacteria.

3.6.3.2. Catalase test

A few drops of three percent hydrogen peroxide were placed at the center of the sterile glass slide and a loopful of bacterial inoculum was agitated in the solution and observed for formation of effervescence (Cappucino and Sherman, 1992).

3.6.3.3. Citrate utilization test

Bacterial isolates were inoculated into test tube containing Simmon's citrate agar medium (Seeley and Vandemark, 1981) (Appendix VI) and incubated for 24 to 48 h. Presence of growth and colour change were noticed.

3.6.3.4. Hydrogen sulphide production

Sulphide indole motility (SIM) agar (Appendix VII) deep tubes were stab inoculated with bacterial isolates and incubated at room temperature for 24 to 48h and observed for the colour development.

3.6.3.5. Growth on tryptone glucose broth

Tryptone glucose broth (Appendix VIII) (Seeley and Vandemark, 1981) was dispensed in 5 ml quantities in test tubes and autoclaved. The tubes were inoculated with 0.1 ml of 24 h old bacterial isolates and incubated at room temperature and observed for the turbidity.

3.6.3.6. Arginine dihydrolase reaction

Thornley's medium (Appendix IX) was used for the study (Thornley, 1960). Five ml aliquots, of the semisolid medium was dispensed in test tubes, autoclaved, cooled and stabbed with test isolates. The surface of the medium was sealed with sterile liquid paraffin to a depth of 1cm. The tubes were incubated at room temperature and observations were recorded for seven days at regular intervals. A change in the colour of the medium to red indicated arginine hydrolase activity.

3.6.3.7. Starch hydrolysis

Nutrient agar containing 0.2 percent soluble starch was employed for this test. The test isolates were spot inoculated on the medium poured in sterilized Petri dishes. Starch hydrolysis was tested after four days of incubation, by flooding the agar surface with Lugol's iodine solution. A colourless zone around the bacterial growth in contrast to the blue background indicated positive starch hydrolysis.

3.6.3.8. Nitrate reduction test

Nitrate broth medium (Appendix X) was used for the test. The medium was dispensed in tubes, autoclaved, inoculated with different isolates of bacterium, incubated, and tested for the reduction of nitrate at regular intervals up to 15 days.

The test was performed by adding few drops of Griess Ilosvay's reagent consisting of sulphanilic acid (0.8 per cent in 5M acetic acid) to the nitrate broth culture. If no pink or red colour developed, it indicated that nitrate was present as such or reduced to ammonia and free nitrogen.

3.6.3.9. Motility test

Bacterial cultures were inoculated on 5ml nutrient broth taken in test tube and incubated at room temperature for 48 h. Then a drop of the diluted culture in sterile water was put on a cavity slide and the movement was observed under a microscope.

3.6.3.10. Presence of fluorescence

Bacterial isolates were streaked on King's B medium (Appendix XI) and incubated at room temperature (28 ± 2 ^{0}C) for 24 to 48 h. Followed by incubation, the bacterial isolates were exposed to UV rays and observed for fluorescence.

3.6.4. Identification using Hi Assorted biochemical test kit

Besides the above biochemical tests, Hi Assorted Biochemical test kit (HIMEDIA® Laboratories Pvt. Ltd., Mumbai) was also used for the identification of selected bacterial isolates. The biochemical test kit was a standardized colorimetric identification system utilizing seven conventional biochemical tests and five carbohydrate utilization tests. The tests were based on the principle of pH change and substrate utilization. On incubation, organisms undergo metabolic changes that were indicated by a colour change in the media that were interpreted either visually or after addition of the reagent. Result interpretation chart is presented in Table 2.

TABLE 2. CHARACTERIZATION OF BACTERIA USING BIOCHEMICAL TEST KIT

Sl.No.	Test	Reagent to be added after incubation	Principle	Original colour of the medium	Positive reaction	Negative reaction
1	Citrate utilization	--	Detects capability of organism to utilize citrate as a sole carbon source	Yellowish-green	Blue	Yellowish-green
2	Lysine decarboxylase	--	Detects lysine decarboxylation	Olive green	Purple	Yellow
3	Ornithine decarboxylase	--	Detects Ornithine decarboxylation	Olive green	Purple	Yellow
4	Urease	--	Detects urease activity	Orangish yellow	Pink	Orangish yellow
5	Phenylalanine Deamination	2-3 drops of TDA reagent	Detects Phenylalanine Deamination activity	Colourless	Green	Colourless
6	Nitrate reduction	1-2drops of sulphanilic acid and 1-2drops of N,N-Dimethyl-1-Napthylamine	Detects Nitrate reduction	Colourless	Pinkish Red	Colourless
7	H_2S production	--	Detects H_2S production	Orangish yellow	Black	Orangish yellow
8	Glucose	--	Glucose utilization	Red	Yellow	Red / pink
9	Adonitol	--	Adonitol utilization	Red	Yellow	Red / pink
10	Lactose	--	Lactose utilization	Red	Yellow	Red / pink
11	Arabinose	--	Arabinose utilization	Red	Yellow	Red / pink
12	Sorbitol	--	Sorbitol utilization	Red	Yellow	Red / pink

3.7. EXTRACTION OF BIOSURFACTANT FROM THE SELECTED BACTERIA

Biosurfactant was extracted from the selected bacterial isolates using the method suggested by Cameotra (1995). Five ml of 24 h old bacterial culture broth was inoculated into 100 ml of ISM broth (involving 0.5 per cent glucose and 1 ml cyclohexane) in 250 ml conical flasks. The flasks were incubated for five days over a shaker set at 125 strokes per min. After the incubation, the culture broth was centrifuged at 10,000 xg for 30 min and the clear supernatant was collected after discarding the cells in the bottom of centrifuge tube. Volume of the clear supernatant was reduced to one fifth by evaporation. The condensed filtrate was added with three volumes of chilled acetone for precipitation of biosurfactant. After addition of acetone, the flasks were placed in a refrigerator for 12 h. This was again centrifuged at 5000 xg for 10 minutes. The supernatant was discarded and the residue was collected in vials in 5 ml of 80 per cent methanol. Crude biosurfactant was quantified and stored in the refrigerator.

3.8. ESTIMATION OF HYDROCARBON EMULSIFYING ACTIVITY OF BIOSURFACTANT BY XYLENE EMULSIFICATION ASSAY

This estimation was done following the method by Banat *et al.* (1991). Five ml 20 mM Tris Buffer (pH 8.0), 1ml of crude biosurfactant sample from eight BS isolates and 1 ml xylene were added in a clean glass test tube. The mixture was vortexed at high speed for 2 min and allowed to sit at room temperature ($28 \pm 2\ ^{0}C$) without further agitation. The absorbance value was read in a spectrophotometer at 610 nm after 1 h and at 24h of incubation period. High absorbance value indicates a high level of dispersion of the xylene in the buffer.

3.9. ESTIMATION OF SURFACE TENSION OF LIQUIDS BY THE ACTIVITY OF BIOSURFACTANT BY DROP WEIGHT METHOD

Drop weight method suggested by Narasimhan *et al.* (1961) was adopted to measure the surface tension of liquid as influenced by biosurfactant activity. To measure the surface tension of liquids, a quantity of 10ml of distilled water, glycerol (10^{-1}dilution), cyclohexane and methoxyethanol monomethyl ether were taken in clean beakers.

To each beaker, 1ml quantity of biosurfactant from eight bacterial isolates were added and mixed with glass rode. The solution was mounted on a 25 ml burette and the flow rate of the liquid was adjusted to ten drops per minute. Ten drops were collected in a pre-weighed beaker and mass of single drop was calculated by carefully weighing the beaker with liquid. Appropriate controls were also maintained (liquid without the addition of biosurfactant). Using a screw-gauge, the radius of the burette nozzle was determined.

Surface tension of the liquid was calculated by the following formula,

$$\text{Surface tension (ST)} = \frac{m \times g}{3.8 \times r} \quad \text{N/m}$$

Where, m = mass of single drop (kg)

g = 9.8 m/s^2 (acceleration due to gravity)

r = radius of the tip of the burette (m)

3.10. EFFECT OF NUTRITIONAL AND CULTURAL CONDITIONS ON PRODUCTION AND EMULSIFICATION ACTIVITY OF BIOSURFACTANTS

Based on the quantity of biosurfactants obtained from the eight bacterial isolates, the most effective isolates *viz;* MCN-3. KFS1, DTSC3 and KFN2 were selected to estimate the effect of nutritional and cultural conditions on the BS production and emulsification activity.

3.10.1. Effect of sugar sources on the biosurfactant production and emulsification activity

100 ml each of ISM broth was prepared separately in 250ml conical flask and autoclaved. Different sugar sources *viz*; sucrose, maltose, mannitol and glucose of 0.5 per cent were prepared separately and sterilized by tyndalization and were added to sterilized ISM broth. Five ml culture broth of the four bacterial isolates were inoculated into the 100 ml ISM broth containing 1ml cyclohexane. The broth was incubated for five days at room temperature. The biosurfactants were extracted from this broth and xylene emulsifying activity was carried out.

3.10.2. Effect of hydrocarbon sources on the biosurfactant production and emulsifying activity

100 ml of ISM broth was prepared separately in 250 ml conical flask and sterilized. 0.5 per cent sterilized glucose was then added to ISM broth. Five ml each culture broth of the bacterial isolates was inoculated into ISM broth containing 1 ml of different hydrocarbon sources viz., cyclohexane, xylene, neem oil and kerosene. The broth was incubated at room temperature for five days in an orbital shaker. From

each sample biosurfactants were extracted and xylene emulsification activity were assessed.

3.10.3. Effect of pH on biosurfactant production and emulsifying activity

100 ml ISM broth of different pH 5, 6, 7 and 8 were sterilized and 0.5 per cent sterilized glucose was then added to ISM broth. Five ml culture broth of the bacterial isolates was inoculated to the broth containing 1 ml cyclohexane. The broth was incubated at room temperature for five days in an orbital shaker. Biosurfactants were extracted and xylene emulsification activities were estimated.

3.10.4. Effect of temperature on biosurfactant production and emulsifying activity

100 ml ISM broth were sterilized and 0.5 per cent sterilized glucose was then added to ISM broth. Five ml each of culture broth of the bacterial isolates was inoculated into the ISM broth containing 1ml cyclohexane. The broth was incubated at different temperatures 20 ^{0}C, 30 ^{0}C and 40 ^{0}C in a rotary shaker and biosurfactants were extracted and xylene emulsifying activities were estimated.

3.11. STUDIES ON THE DEGRADATION OF PESTICIDES BY BIOSURFACTANT PRODUCING BACTERIA

Based on quantity of BS production and xylene emulsification assay, three BS bacteria MCN-3, KFS1 and DTSC3 were selected for the estimation of pesticide degradation in soil.

3.11.1. Degradation of chlorpyriphos

Degradation of chlorpyriphos in soil was done following the method of Suri and Joia (1996). Hundred gram of well sterilized soil were mixed separately with three selected BS bacterium viz., MCN-3, KFS1, and DTSC3 and incubated at room temperature. The bacterial population was estimated at 24h interval by serial dilution technique (Johnson and Curl, 1972) for assessing the time required for maximum bacterial population. Accordingly, each soil sample was enriched with test bacteria and incubated at room temperature for three days to attain maximum bacterial population. Suitable controls and replications were maintained.

Chlorpyriphos residue analysis was carried out using gas chromatograph (GC). A standard solution (1.396 mg / ml) of chlorpyriphos was applied to each 100 g soil sample, so that the level of chlorpyriphos concentration was obtained as 55.84 μ g / g soil. Each soil sample was mixed well and moisture content was maintained at 23 per cent. From these soil samples, a quantity of 15 g each was taken and mixed with 0.3 g activated charcoal, 2 g florisil and 10 g anhydrous sodium sulphate and then packed in a glass column with 3 cm layer of anhydrous sodium sulphate at both ends. Chlorpyriphos was then eluted out using 10 per cent acetone in hexane by passing through the column. The elutant was collected and concentrated to one ml. Final volume was made up to 10 ml with n-hexane and quantified in Shimadzu Gas Chromatograph 2010 equipped with electron capture detector (ECD). At forty days after application of chlorpyriphos, bacterium enriched soils were analyzed for its residues using gas chromatograph.

3.11.1.1. Conditions for gas chromatograph

Capillary column- BPX5 30m x 0.25 mm ID

3.11.1.2. Temperature conditions

Column	-	220 ^{0}C
Injector	-	250 ^{0}C
Detector	-	280 ^{0}C
Carrier gas	-	Nitrogen
Retention time	-	11.6 min

3.11.2. Degradation of mancozeb

Studies on the degradation of mancozeb was carried out based on the procedure suggested by Keppel (1971).

3.11.2.1. Standardization of procedure for determination of mancozeb residue

Mancozeb in presence of sulphuric acid (10N) is decomposed into carbon disulphide (CS_2). So, before assessing the residue of mancozeb in soil, standardization of the method is to be done for estimating the actual concentration of CS_2 present in the residue of mancozeb.

a) Preparation of standard CS_2 solution

One gram of CS_2 was weighed into a 25 ml beaker containing 15 ml of ethanol and transferred quantitatively in to a 100 ml volumetric flask using ehanol and made up the volume.

b) Preparation of standard curve

In a series of 25 ml standard flasks, required amount of CS_2 standard solution was taken and 12.5 ml colour reagent (0.004 g cupric acetate monohydrate dissolved into 25 g of diethanolamine in ethanol and made up the volume to 250 ml) was added and made up the volume with ethanol so as to get 100 ppm, 50 ppm, 10 ppm, 5 ppm and 1 ppm standard solutions. These solutions were allowed to stand for 15 min and the absorbance was measured at 435 nm using spectrophotometer. These values were plotted against concentrations.

3.11.2.2. Residue analysis of Mancozeb

A quantity of 100 g dried and well sterilized soil samples were enriched separately with the test bacteria viz., MCN-3, KFS1 and DTSC3 and incubated at room temperature for three days to attain maximum population. The soil samples were drenched with 0.4 per cent mancozeb (75 % WP). Suitable check and replications were maintained.

Mancozeb residues of the soil samples were analyzed using the decomposition absorption apparatus on 5^{th}, 10^{th} and 40^{th} days after application of fungicide. Water was allowed to flow through the condenser of the apparatus. Out of the two traps contained in the apparatus, the first trap was filled with 15 ml of 20 per cent zinc acetate solution and the other one with 12.5 ml colour reagent. A quantity of 100 g soil sample was introduced into the dry reaction flask through the 35 / 25 ball joint. The weight of the sample was adjusted so that the total dithiocarbamate (DTC) residue was equivalent to 50 - 100 μ g of CS_2. A funnel was placed in the 35 / 25 ball joint and 150 ml of 10 N sulphuric acid was added to it. The tap connected to water pump was opened and adjusted the air inlet, so that air swept in the system @ 6-10 ml

per min. The content of the flask was heated immediately and the digestion continued for 45 min so as to complete the decomposition of mancozeb in to ethylene thio urea (ETU) and CS_2. The second trap containing CS_2 was transferred quantitatively to a 25 ml volumetric flask using ethanol and made up the volume with ethanol. The solution was kept for 15 min and the absorbance was measured at 435 nm. Using the K factor obtained from the standardardization the amount of CS_2 was estimated and the quantity of mancozeb was computed using the equation,

$$\mu \text{ g of mancozeb} = \mu \text{ g of } CS_2 \times 1.75$$

3.12. *In vitro* ANTIMICROBIAL ACTIVITY OF SELECTED BIOSURFACTANT PRODUCING BACTERIA AGAINST SOIL BORNE PATHOGENS AND BIOCONTROL AGENTS

3.12.1. *In vitro* Antagonistic effect of biosurfactant producing bacteria against selected soil borne pathogens

Effect of eight selected BS bacteria against soil borne pathogens like *Pythium aphanidermatum, Phytophthora capsici* and *Rhizoctonia solani* were studied under *in vitro* condition by dual culture method.

3.12.1.1. Streaking on one side

Mycelial discs of 6mm size of test pathogen viz., *P. aphanidermatum, P. capsici* and *R. solani* were inoculated on one side of a potato dextrose agar medium (PDA) (Appendix XII) in Petri plate and incubated at room temperature for 24h. After this, the test bacterial isolate was streaked as a line in the same Petri dish 2.5cm away from the pathogen disc and incubated. Three replications were

maintained for each treatment. The pathogen grown in monoculture served as controls. Suitable controls were maintained. Growth of test fungus was recorded daily till the control plate fully covered.

3.12.1.2. Streaking on both sides

Mycelial discs of 6mm size of soil borne pathogens viz., *P. aphanidermatum, P. capsici* and *R. solani* were inoculated at the centre of PDA medium in Petri plate 24h prior to the inoculation of the bacteria and incubated at room temperature. The bacterial isolates to be tested were inoculated as a line of streak on either side of the pathogen 2.5cm away from the disc. A control plate of fungal pathogen was also maintained without streaking bacteria. Growth of test fungus was recorded daily till the control plate was fully covered with the fungal growth.

Per cent inhibition of growth over control was calculated by the formula suggested by Vincent (1927),

$$\text{Per cent inhibition of growth} = \frac{C - T}{C} \times 100$$

Where, C = Growth of fungus in control (mm)

T = Growth of fungus in treatment (mm)

3.12.2. Compatibility studies of selected BS producing bacteria with biocontrol agents

Compatibility of BS bacteria with *Pseudomonas fluorescens, Trichoderma harzianum* and *T. viride* were studied by using standard protocols.

3.12.2.1. Pseudomonas fluorescens

a) Cross streaking method

On nutrient agar medium, both the biosurfactant producing bacterium and standard culture of *Pseudomonas fluorescens* were streaked perpendicular to each other and incubated at room temperature. Plates were observed for the lysis at the juncture of both test and the indicator organism. Three replications were maintained for each BS bacteria.

b) Point inoculation method

Standard culture discs of *P. fluorescens* (6 mm size) were inoculated on nutrient agar medium seeded with BS bacteria and incubated at room temperature (28 ± 2 ^{0}C). Plates were observed till 48 h and the diameter of inhibition zone was recorded.

3.12.2.2. Trichoderma harzianum and *T. viride*

Compatibility of BS bacteria with *T. harzianum* and *T. viride* were studied by adopting dual culture methods as mentioned in 3.12.1.1. and 3.12.1.2.

3.13. EFFECT OF SELECTED BIOSURFACTANT PRODUCING BACTERIA ON PLANT GROWTH CHARACTERISTICS

Eight promising BS isolates were screened for their effect on plant growth characters by testing per cent seed germination and other growth parameters viz., radicle and plumule length using the standard procedures. The effect of BS bacterium on seed germination were tested in both sorghum and cowpea seeds. The seeds were

surface sterilized with 0.2 per cent sodium hypo chlorite for three min followed by successive washings with sterile distilled water. The water decanted and the seeds were treated with 48 h old BS bacterial cultures for 10 min.

In the meantime, 0.8 per cent plain agar medium was poured into the sterile Petri plates and seeds were placed on the solidified medium and incubated at room temperature for two to three days. Suitable controls and replications were maintained. Germination per cent of seeds were observed 24 h after incubation where as, root length and shoot length were recorded at three days after incubation.

3.14. STATISTICAL ANALYSIS

Analysis of variance was performed on the population studies of total heterotrophic bacteria and the effect of nutritional and cultural conditions on production and emulsifying activity of biosurfactant using the statistical package MSTATC (Freed, 1986). In the experiment on the effect of BS bacteria on plant growth characters of cowpea and sorghum seeds, the data were compared using Duncan's multiple range test (DMRT).

4. RESULTS

The results of the experiments on "Biosurfactant producing bacteria from selected soils of Kerala" are presented in this chapter.

4.1. ISOLATION OF HETEROTROPHIC BACTERIA FROM SELECTED SOIL SAMPLES

A total of 92 bacterial isolates were obtained from the ten soil samples collected from different hydrocarbon contaminated locations (Plate I). Population of heterotrophic bacteria in hydrocarbon contaminated soil samples are presented in Table 3 (Fig. 1). Bacterial populations among the soil samples were differed significantly ($2.33 \times 10^7 - 124.0 \times 10^7$ cfu / g soil).

Sample enrichment showed significant difference among each other. A significant increase in bacterial population was observed in neem oil and crude oil enriched soil samples. Neem oil registered as the best source of enrichment material compared to crude oil and non enriched soil samples.

In neem oil enriched samples, maximum population was recorded by the soil samples from chlorpyriphos treated plot (124.0×10^7 cfu / g soil), followed by herbicide treated plot - Location 2 (79.67×10^7 cfu / g soil). When soil samples were enriched with crude oil, the bacterial population was found to be maximum in chlorpyriphos treated plot (100.67×10^7 cfu / g soil) followed by herbicide treated plot - Location 2 (74.67×10^7 cfu / g soil).

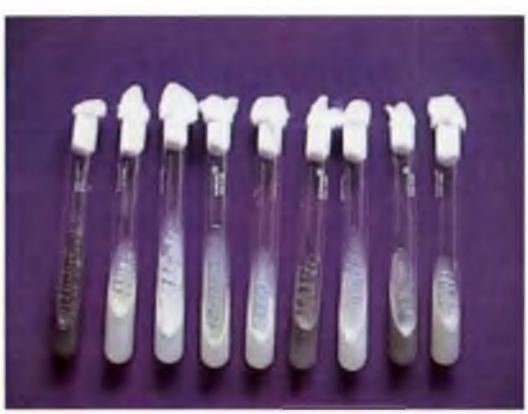
The soils without enrichment recorded a maximum bacterial population of 53.33×10^7 cfu / g from Maruti automobile service station (MSP-2) followed by

PLATE - I.

Isolation of heterotrophic bacteria from soils of Kerala

a. Enrichment of soil samples

b. Heterotrophic bacterial colonies



c. Pure cultures of bacterial isolates

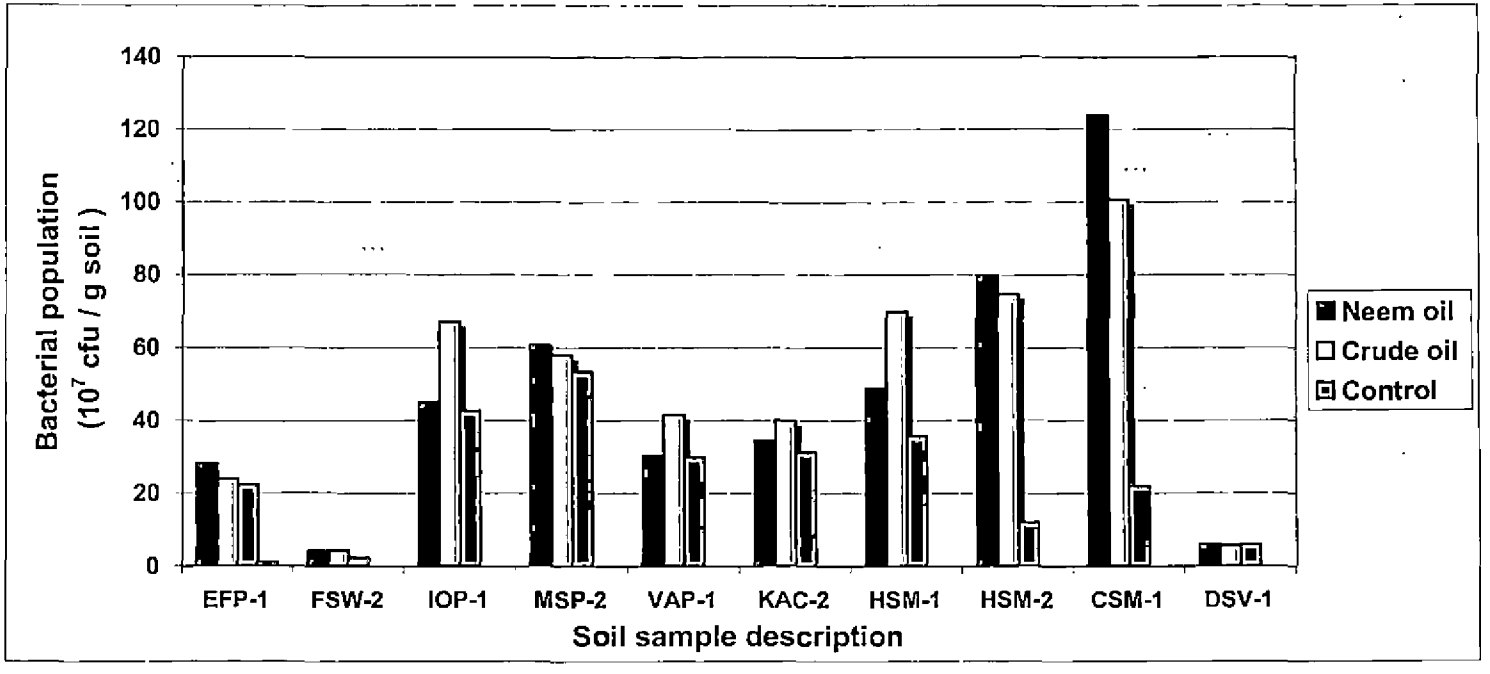
Table 3 Population of heterotrophic bacteria in hydrocarbon contaminated soil samples

Sl.No.	Soil sample description	Microbial population (10^7 cfu/g soil)		
		Samples enriched with		Without enrichment (Control)
		Neem oil	Crude oil	
1	*Eucalyptus* plantations, Kerala Forest Research Institute, Peechi (EFP-1)	28.33 (5.37)	24.00 (4.94)	22.33 (4.78)
2	Forest lands, Wyanad (FSW-2)	4.33 (2.19)	4.33 (2.20)	2.33 (1.68)
3	Indian Oil Petroleum Bunk, Peringottukara, Thrissur (IOP-1)	45.00 (6.75)	67.00 (8.22)	42.67 (6.57)
4	Maruti automobile service station, Pazuvil, Thrissur (MSP-20)	60.67 (7.82)	58.00 (7.65)	53.33 (7.34)
5	Vydyaratnam Ayurveda Pharmacy, Perumbilllisery , Thrissur (VAP-1)	30.33 (5.52)	41.67 (6.50)	30.00 (5.52)
6	Kunnathuvalappil Ayurveda Nursing Home, Cherpu, Thrissur (KAC-2)	34.33 (5.90)	40.00 (6.36)	31.33 (5.64)
7	Herbicide treated plot, Agricultural Research Station, Mannuthy -Location 1 (HSM-1)	48.67 (7.01)	70.00 (8.40)	35.67 (6.01)
8	Herbicide treated plot, Agricultural Research Station, Mannuthy -Location 2 (HSM-2)	79.67 (8.95)	74.67 (8.67)	12.00 (3.53)
9	Chlorpyriphos treated plot, Mannarkad, Palakkad (CSM-1)	124.00 (11.16)	100.67 (10.05)	22.00 (4.70)
10	Mancozeb treated vegetable plot, Horticulture College,Vellanikkara (MSV-1)	6.00 (2.53)	5.67 (2.47)	6.00 (2.53)
	Mean	51.43 (6.68)	35.23 (5.54)	33.83 (5.47)

Figures in parentheses are $\sqrt{x}$ +0.05 transformed values

$CD_{(0.05)}$ for soils -- 0.244 $CD_{(0.05)}$ for enrichment -- 0.134

Fig. 1. Population of heterotrophic bacteria in hydrocarbon contaminated soil samples



EFP-1	*Eucalyptus* plantations, Kerala Forest Research Institute, Peechi
FSW-2	Forest lands, Wyanad
IOP-1	Indian oil petroleum bunk, Peringottukara, Thrissur
MSP-2	Maruti service station, Pazhuvil, Thrissur
VAP-1	Vydyaratnam ayurveda nursing home, Perumbillisery, Thrissur
KAC-2	Kunnathuvalappil ayurveda nursing home, Cherpu, Thrissur
HSM-1	Herbicide treated plot-Location 1, Mannuthy
HSM-2	Herbicide treated plot-Location 2, Mannuthy
CSM-1	Chlorpyriphos treated plot, Mannarkad
	cozeb treated vegetable plot, Vellanikkara

Indian oil petroleum bunk (IOP-1) (42.67 x 10^7 cfu / g soil). Where as in mancozeb treated vegetable plot (MSV-1) and in *Eucalyptus* plantations (EFP-1) the bacterial population was almost same even after enrichment.

4.2. SCREENING OF BACTERIAL ISOLATES FOR SURFACTANT PRODUCTION ACTIVITY

4.2.1. Drop collapse technique

In drop collapse technique, the bacteria that produced BS soon collapsed and spread immediately on neem oil coated plate, where as the other isolates which did not produce BS remained as uncollapsed (plate II A). The area of dispersion was recorded and presented in Table 4.

Out of the 92 bacterial isolates, only 36 isolates gave positive reaction to drop collapse assay. Among these 36 isolates, the isolate KFN2 (*Eucalyptus* plantations) gave the maximum area of dispersion (333 mm^2) followed by DTSC3 isolate (324.60 mm^2) from mancozeb treated vegetable plot and MCN-3 isolate (310 mm^2) from chlorpyriphos treated plot. A moderate area of dispersion of bacterial culture drops was showed by other isolates viz., KFS1 (276.6 mm^2), MCC-2 (251.60 mm^2), DTSC5 (218.50 mm^2), PFC-4 (170.66 mm^2) and KCC-2 (170 mm^2) respectively. The bacteria isolated from automobile service station did not give notable area of dispersion.

4.2.2. Xylene spray method

Xylene spray method was also used for the detection of BS producing bacterial isolates. In this method, only 24 isolates gave positive results (Table 5). Among the 24 isolates, eight isolates were recorded a good area of clear zone varying

Table 4. Screening for biosurfactant producing bacteria by drop collapse technique

Soil sample	Bacterial isolates	Area of the bacterial culture drop*		
		Initial area (mm^2)	Final area (mm^2)	Area of dispersion (mm^2)
i	*Eucalyptus* plantations, Peechi			
	1) KFN2	25	358.0	**333.0**
	2) PFC-4	25	195.66	**170.7**
	3) KFS1	25	301.6	**276.6**
ii	Forest lands, Wyanad			
	4) FWN-1	25	94.6	69.60
	5) FWN-2	25	72.6	47.60
	6) FWN-3	25	34.0	9.00
	7) FWS-1	25	51.3	26.30
iii	Indian Oil Petroleum Bunk, Peringottukara			
	8) IPS-1	25	100.0	75.00
	9) IPS-2	25	72.6	47.60
	10) IPS-3	25	72.3	47.30
iv	Maruti automobile service station, Pazuvil			
	11) MPC-3	25	60.6	35.60
	12) MPN-1	25	29.0	4.00

(Continued)

Soil sample	Bacterial isolates	Area of the bacterial culture drop*		
		Initial area (mm^2)	Final area (mm^2)	Area of dispersion (mm^2)
v	Vydyaratnam Ayurveda Pharmacy, Perumbilllisery			
	13) VPC-1	25	32.6	7.60
vi	Kunnathuvalappil Ayurveda Nursing Home, Cherpu			
	14) KCC-2	25	195.0	**170.0**
	15) KCS-1	25	52.6	27.60
vii	Herbicide treated plot, Agricultural Research Station, Mannuthy (Location 1)			
	16) HMC-6	25	96.6	71.60
	17) HMS-3	25	34.16	9.160
	18) HMS-6	25	78.6	53.60
viii	Herbicide treat ed plot, Agricultural Research Station, Mannuthy (Location 2)			
	19) H_2MN-3	25	166.6	141.60
	20) H_2MN-2	25	45.6	20.60
	21) H_2MC-6	25	125.0	100.00
	22) H_2MS-6	25	81.3	56.30

(Continued)

Soil sample	Bacterial isolates	Area of the bacterial culture drop*		
		Initial area (mm^2)	Final area (mm^2)	Area of dispersion (mm^2)
ix	Chlorpyriphos treated plot, Mannarkad			
	23)MCN-3	25	335.0	**310.00**
	24) MCC-2	25	276.6	**251.60**
	25) MCS-2	25	52.0	27.00
	26) MCS-4	25	51.6	26.60
x	Mancozeb treated vegetable plot, Vellanikkara			
	27) DTS C-5	25	243.5	**218.50**
	28) DTS C3	25	349.6	**324.60**
	29) DTS C4	25	64.6	39.60
	30) DTS S2	25	83.16	58.16
	31)DTS C2	25	72.0	47.00
	32) DTS S3	25	50.6	25.60
	33) DTS N1	25	71.3	46.30
	34) DTS C1	25	73.6	48.60
	35) DTS S1	25	107.5	82.50
	36) DTS N2	25	81.16	56.16
	CONTROL (Uninoculated broth)	25	25	0

*Mean of three replications

from 289.38-961.62 mm^2 . However, KFN2 gave the maximum area of clear zone (961.62 mm^2) followed by the isolates DTSC5 and MCC-2. A moderate area of dispersion (289.38 mm^2 – 783.86 mm^2) were recorded by the isolates viz., KCC-2 (Kunnathuvalappil ayurveda nursing home), PFC-4, KFS1 (*Eucalyptus* plantations); MCN-3 (chlorpyriphos treated plot) and DTSC3 (mancozeb treated vegetable plot) respectively (Plate II B).

In xylene spray method also, isolates from Maruti automobile service station, Indian Oil Petroleum Bunk and Forest lands of Wyanad did not show any noticeable result. However, in case of herbicide treated plot an appreciable result was noticed with xylene spray method. On comparison, maximum area of dispersion was obtained with KFN2 isolate in both methods. Xylene spray method is found to be better than drop collapse method as it yielded maximum area of dispersion.

4.3. SELECTION OF BIOSURFACTANT PRODUCING BACTERIA

Based on the two screening tests viz., drop collapse and xylene spray methods, the most effective eight BS producing bacterial isolates were selected. They are,

1. KCC-2 (Kunnathuvalappil Ayurveda Nursing Home, Cherpu, Thrissur)
2. MCC-2 (Chlorpyriphos treated plot, Mannarkad, Palakkad)
3. MCN-3 (Chlorpyriphos treated plot, Mannarkad, Palakkad)
4. KFS1 (*Eucalyptus* plantations, Kerala Forest Research Institute, Peechi)
5. KFN2 (*Eucalyptus* plantations, Kerala Forest Research Institute, Peechi)
6. PFC - 4 (*Eucalyptus* plantations, Kerala Forest Research Institute, Peechi)
7. DTSC3 (Mancozeb treated vegetable plot, College of Horticulture, Vellanikkara)
8. DTSC5 (Mancozeb treated vegetable plot, College of Horticulture, Vellanikkara)

Table 5. Screening for biosurfactant producing bacteria by xylene spray method

Soil sample	Bacterial isolates	Diameter of clear zone (mm)*	Area of clear zone (mm^2)
I	*Eucalyptus* plantations, Peechi		
	1) KFN-2	35.0	**961.62**
	2) PFC-4	19.3	**289.38**
	3) KFS-1	28.0	**615.44**
ii	Forest lands, Wyanad		
	4) FWN-1	20.0	**314.0**
	5) FWN-2	11.0	94.98
	6) FWN-3	2.66	5.55
	7) FWS-1	10.33	83.60
iii	Indian Oil Petroleum Bunk, Peringottukara		
	8) IPS-1	12.3	118.70
	9) IPS-2	--	--
	10) IPS-3	--	--
iv	Maruti automobile service station, Pazuvil		
	11) MPC-3	10.66	89.20
	12) MPN-1	--	--
v	Vydyaratnam Ayurveda Pharmacy, Perumbillliscry		
	13) VPC-1	--	--
vi	Kunnathuvalappil Ayurveda Nursing Home, Cherpu		
	14) KCC-2	19.3	**289.38**
	15) KCS-1	17.0	226.86
vii	Herbicide treated plots, Agricultural Research Station, Mannuthy (Location 1)		

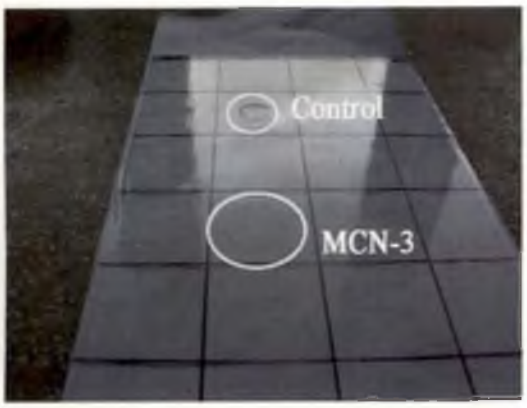
(Continued)

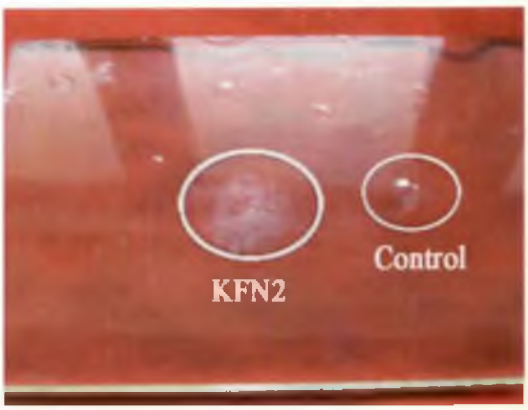
Soil sample	Bacterial isolates	Diameter of clear zone (mm)*	Area of clear zone (mm^2)
	16) HMC-6	14.3	160.50
	17) HMS-3	--	--
	18) HMS-6	19	283.38
viii	Herbicide treat ed plots, Agricultural Research Station, Mannuthy (Location 2)		
	19) H_2MN-3	14.0	153.86
	20) H_2MN-2	--	--
	21) H_2MC-6	15.6	192.50
	22) H_2MS-6	17.33	235.48
ix	Chlorpyriphos treated plots, Mannarkad		
	23) MCN3	28.6	642.09
	24) MCC-2	32.6	834.26
	25) MCS-2	--	--
	26) MCS-4	--	--
x	Mancozeb treated vegetable plots, Vellanikkara		
	27) DTS C-5	33.0	854.86
	28) DTS C3	31.6	783.86
	29) DTS C4	15.0	176.62
	30) DTS S2	--	--
	31)DTS C2	--	--
	32) DTS S3	--	--
	33) DTS N1	--	--
	34) DTS C1	--	--
	35) DTS S1	11.3	100.23
	36) DTS N2	19.0	125.81

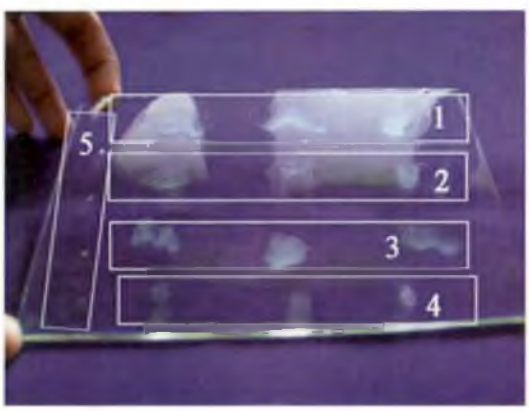
*Mean of three replications

PLATE - II

A. Drop collapse technique for screening biosurfactant bacteria

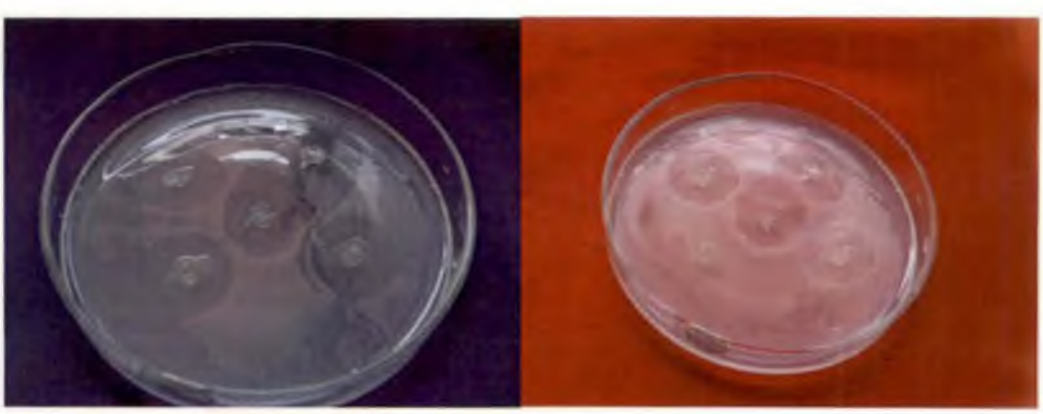






1.KFN2
2. KFS1
3. MCN-3
4. KFC4
5. Control

B.Xylene spray method for screening BS bacteria



Biosurfactant producing bacterial colonies

4.4. ESTIMATION OF BIOSURFACTANT PRODUCING BACTERIAL POPULATION IN SELECTED SOIL SAMPLES

Among the heterotrophic bacterial isolates obtained from the selected soil samples, number of BS producing isolates was worked out by the screening tests. From this, per cent BS bacterial population in the soil samples was calculated and the results are presented in Table 6 (Fig. 2a and 2b). Among the ten soil samples estimated, maximum BS bacterial population of 45.4 per cent was found in mancozeb treated vegetable plot, Vellanikkara. Samples from *Eucalyptus* plantations, Peechi and forest lands, Wyanad scored 37.5 per cent BS bacteria followed by herbicide treated plot-Location 2, Mannuthy (36 per cent). Soils of Kunnathuvalappil Ayurveda Nursing home, herbicide treated plot-Location 1 Mannuthy and chlorpyriphos treated plot, Mannarkad were recorded 25 per cent BS bacteria. Maruti Service station, Pazhuvil and Indian Oil Petroleum bunk, Peringottukara recorded BS bacteria of 12.5 per cent and 8.3 per cent respectively. Soil samples from Vydyaratnam Ayurveda Nursing home, Cherpu, did not record BS bacterial population.

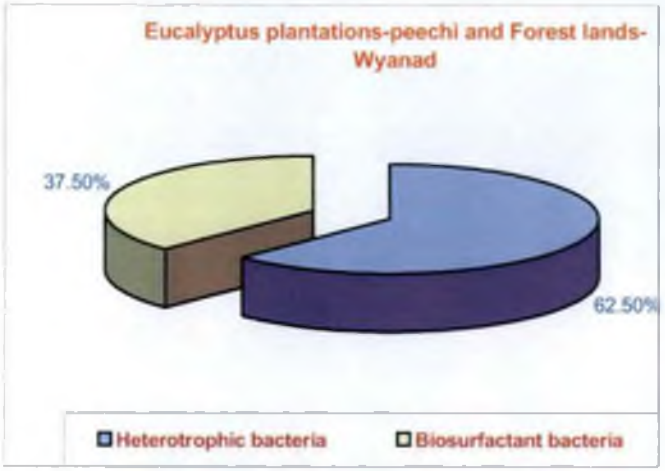
4.5. CHARACTERIZATION OF SELECTED BACTERIAL ISOLATES

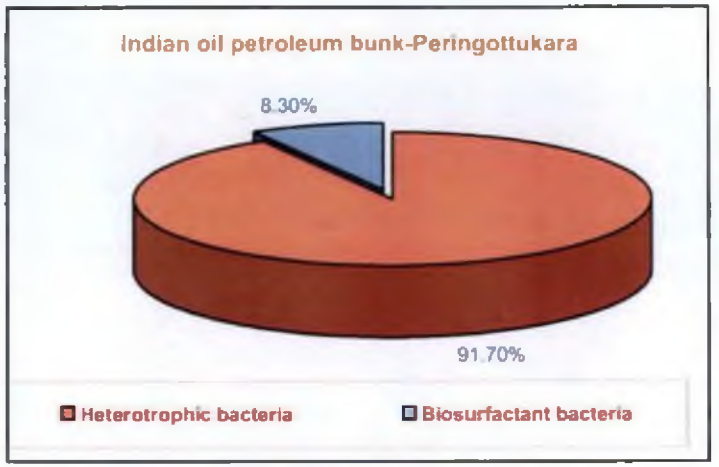
The selected BS bacteria were tentatively identified based on cultural, morphological and biochemical characters (Plate III). Results of the colony morphology and cultural characters are presented in the Table 7.

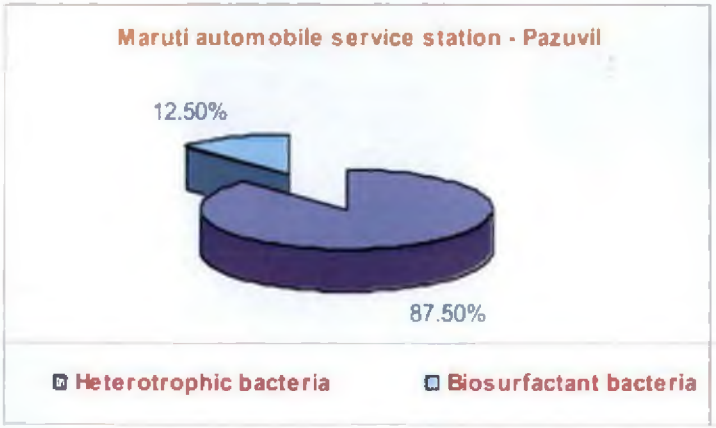
Table 6 Estimation of biosurfactant producing bacteria in selected soil samples

Sl. No.	Source of soil sample	Total no. of bacterial isolates	No. of biosurfactant producing bacteria	Per cent of biosurfactant producing bacteria
1	*Eucalyptus* plantations, Kerala Forest Research Institute, Peechi (EFP-1)	8	3	**37.5**
2	Forest lands, Wyanad (FSW-2)	8	3	**37.5**
3	Indian Oil Petroleum bunk, Peringottukara, Thrissur (IOP-1)	12	1	8.3
4	Maruti automobile service station, Pazuvil, Thrissur (MSP-2)	8	1	12.5
5	Vydyaratnam Ayurveda Pharmacy, Perumbilllisery , Thrissur (VAP-1)	10	0	0
6	Kunnathuvalappil Ayurveda Nursing Home, Cherpu, Thrissur (KAC-2)	8	2	25
7	Herbicide treated plot, Agricultural Research Station, Mannuthy (Location 1) (HSM-1)	8	2	25
8	Herbicide treated plot, Agricultural Research Station, Mannuthy (Location 2) (HSM-2)	11	3	36
9	Chlorpyriphos treated plot, Mannarkad, Palakkad (CSM-1)	8	2	25
10	Mancozeb treated vegetable plot, College of Horticulture, Vellanikkara (MSV-1)	11	5	**45.4**

Fig.2a. Per cent of biosurfactant producing bacteria in soil samples







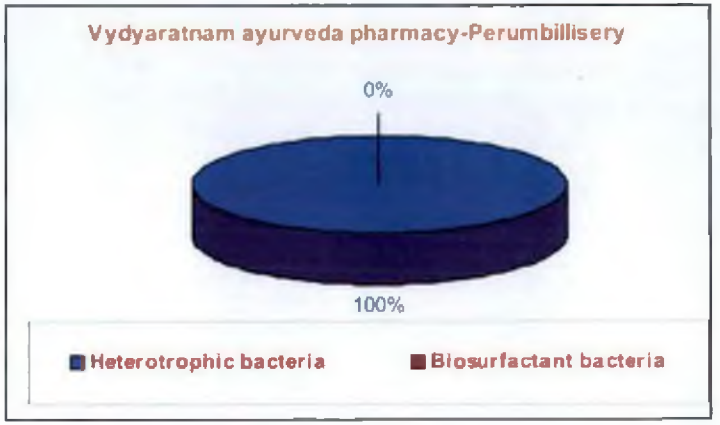
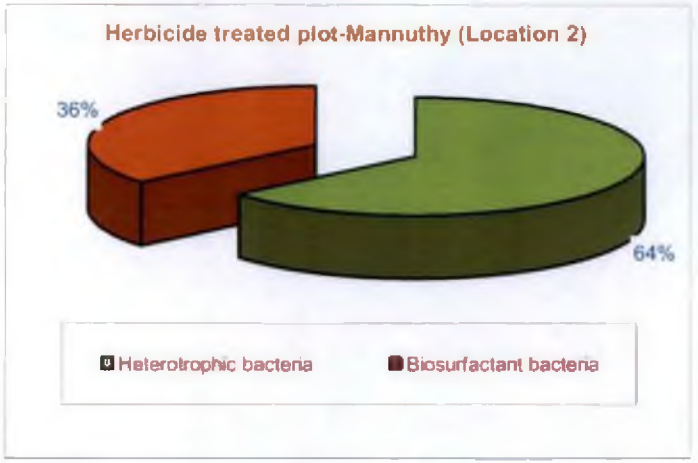
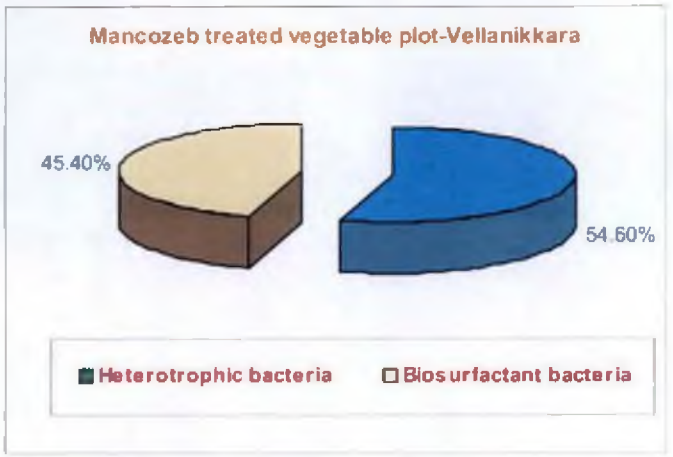
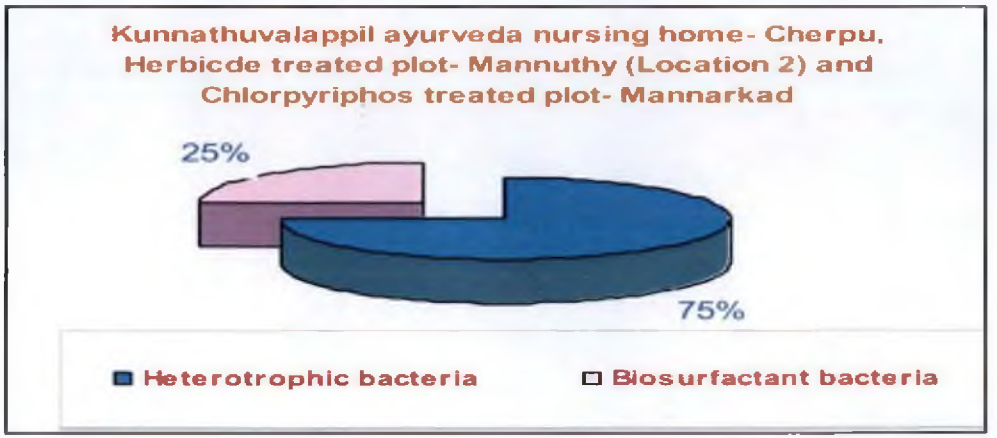


Fig.2b. Per cent of biosurfactant producing bacteria in soil samples







4.5.1. Biochemical characters

4.5.1.1. Potassium hydroxide test

Except MCN-3 isolate, the other seven isolates gave positive reaction with the evidence of thin threads while mixing with 3 per cent KOH indicating gram negative bacteria.

4.5.1.2. Catalase test

The eight selected isolates produced effervescence while mixing with three per cent hydrogen peroxide.

4.5.1.3. Citrate utilization test

Except the isolates MCN-3 and KCC-2 isolate, the others gave positive reaction to this test by giving a colour change of medium from green to blue.

4.5.1.4. Hydrogen sulphide production

The eight isolates did not give any black colouration along the line of stab inoculation indicating negative reaction to this test.

4.5.1.5. Growth on tryptone glucose broth

Among the eight isolates, the isolates, MCC-2, PFC-4, KFN2, KFS1, DTSC3 and DTSC5 were shown good growth on tryptone glucose broth.

Table 7 Colony morphology and Cultural characters of selected biosurfactant producing bacteria

Sl.No.	Characters	BACTERIAL ISOLATES							
		KCC-2	MCC-2	MCN-3	KFS1	KFN2	PFC-4	DTSC3	DTSC5
1	Shape	Circular	Circular	Circular	Circular, round	Circular	Circular	Circular	Circular
2	Margin	Entire	Entire	Entire	Entire	Entire	Entire	Entire	Entire
3	Elevation	Convex	Convex	Raised	Low convex	Raised	Convex	Convex	Convex
4	Surface	Pin head	Slight slimy	Smooth and opaque	Fluidal smooth	Slimy	Smooth and moist	Small to large, slimy	Small to large, slimy
5	Colour	Light yellow to brown	Cream	Cream	Cream	Cream	Cream	Light yellow	Light yellow
6	Gram staining	Gram negative short rods	Gram negative short rods	Gram positive long rods	Gram negative short rods	Gram negative short rods	Gram negative short rods	Gram negative short rods	Gram negative short rods
7	Spore staining	--	--	+	--	--	--	--	--
8	Growth in tryptone glucose broth	Moderate growth	Good well dispersed growth	Moderate growth	Good well dispersed growth	Good well dispersed growth	Good well dispersed growth	Good well dispersed growth	Good well dispersed growth

4.5.1.6. Arginine dihydrolase reaction

Except three isolates KCC-2, MCN-3 and KFN2 all the other isolates gave positive result to this test by changing the colour of Thornley's medium to red, which indicated arginine hydrolase activity.

4.5.1.7. Starch hydrolysis

Among the eight isolates, two isolates, MCN-3 and KCC-2 were given positive reaction with the evidence of white halo surrounding the colony with blue background.

4.5.1.8. Nitrate reduction test

Except the isolate PFC-4, the other seven isolates were changed the colour of medium to pink or red immediately while adding the reagents into the inoculated media.

4.5.1.9. Motility test

Except the isolate MCN-3, the other seven isolates were found to be motile when observed its movement through microscope.

4.5.1.10. Presence of fluorescence

Except MCN-3 and KCC-2 isolates, others produced colour of fluorescence while exposed to UV rays.

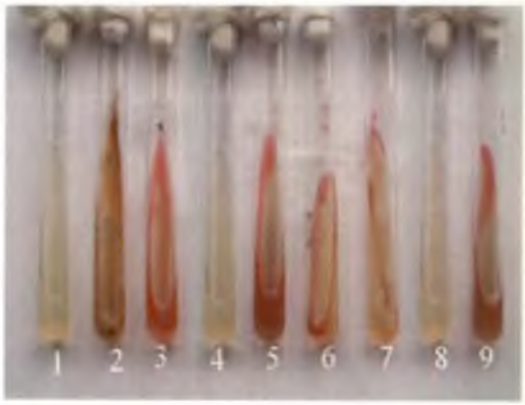
PLATE – III

Biochemical tests of bacterial isolates

a. Citrate utilization

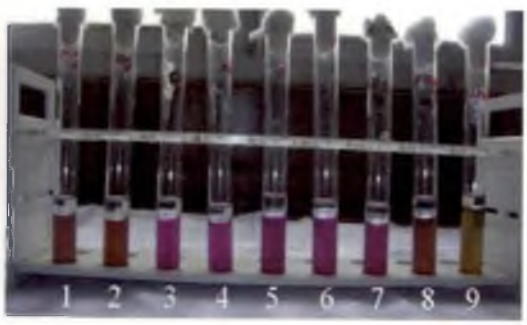
(1) MCC-2
(2) MCN-3
(3)KCC-2
(4)KFS1
(5)KFN2
(6)PFC-4
(7)DTSC3
(8) DTSC5
(9) Control

b. Nitrate reduction



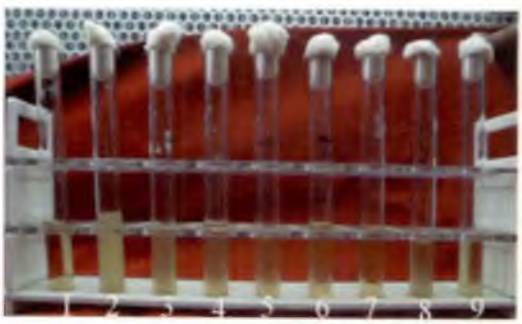
(1) Control
(2) KFS1
(3) KFN2
(4) PFC-4
(5) DTSC3
(6)DTSC5
(7)KCC-2
(8) MCC-2
(9) MCN-3

c. Arginine dihydrolase reaction



(1) KCC-2
(2) MCN-3
(3) MCC-2
(4)KFS1
(5) PFC-4
(6) DTSC3
(7) DTSC5
(8) KFN2
(9) Control

d. Growth on tryptone broth



(1)Control
(2) MCC-2
(3) MCN-3
(4) KFS1
(5) KFN2
(6) PFC-4
(7) DTSC3
(8) DTSC5
(9) KCC-2

4.5.2. Identification of bacterial isolates

1. Isolate KCC-2

Cultural characters- Circular, entire, convex, pinhead and light yellow to brown colony.
Morphological characters- Gram negative short rods
Based on the above cultural and morphological characters and biochemical characters given in table 8, the bacterium is identified as *Pseudomonas* sp.

2. Isolate MCC-2

Cultural characters- Circular, entire, convex, slimy and cream colony.
Morphological characters- Gram negative short rods
Based on the above cultural and morphological characters and biochemical characters given in table 8, the bacterium is identified as *Pseudomonas* sp.

3. Isolate KFS1

Cultural characters- Circular, round, entire, low convex, fluidal smooth and cream coloured colony
Morphological characters- Gram negative short rods
Based on the above cultural and morphological characters and biochemical characters given in table 8, the bacterium is identified as *Pseudomonas* sp.

4. Isolate KFN2

Cultural characters- Circular, entire, raised, cream coloured slimy colony

Table 8. Biochemical characters of selected biosurfactant producing bacteria

Sl.No.	NAME OF TEST	BACTERIAL ISOLATES							
		KCC-2	MCC-2	MCN-3	KFS1	KFN2	PFC-4	DTSC3	DTSC5
9	Catalase	--	+	+	+	+	+	+	+
10	KOH	+	+	--	+	+	+	+	+
11	H_2S production	--	--	--	--	--	--	--	--
12	Citrate utilization	--	+	--	+	+	+	+	+
13	Starch hydrolysis	+	--	+	--	--	--	--	--
14	Arginine dihydrolase	--	+	--	+	--	+	+	+
15	Nitrate reduction	+	--	+	+	+	--	+	+
16	Fluorescens	--	+	--	+	+	+	+	+
17	Motility	+	+	--	+	+	+	+	+
18	Lysine	--	--	--	+	+	--	--	--
19	Ornithine	--	--	+	+	+	--	--	--
20	Urease	--	--	--	--	--	--	--	--
21	TDA	--	--	--	--	--	--	--	--
22	Glucose	--	+	--	+	+	+	+	+
23	Adonitol	--	--	--	--	--	--	--	--
24	Lactose	--	--	--	--	--	--	--	--
25	Arabinose	--	+	--	+	--	+	--	--
26	Sorbitol	--	--	--	+	--	--	--	--
		Pseudomonas sp.	*Pseudomonas* sp.	*Geobacillus kaustophilus* **MTCC 8517**	*Pseudomonas* sp.	*Pseudomonas* sp.	*Pseudomonas fluorescens* **MTCC 8518**	*Pseudomonas* sp.	*Pseudomonas* sp.

+ Positive reaction -- Negative reaction

Morphological characters - Gram negative short rods

Based on the above cultural and morphological characters and biochemical characters given in table 8, the bacterium is identified as *Pseudomonas* sp.

5. Isolate DTSC3

Cultural characters - Circular, entire, convex, light yellow coloured small to large slimy colony

Morphological characters- Gram negative short rods

Based on the above cultural and morphological characters and biochemical characters given in table 8, the bacterium is identified as *Pseudomonas* sp.

6. Isolate DTSC5

Cultural characters- Circular, entire, convex, light yellow coloured small to large slimy colony

Morphological characters- Gram negative short rods

Based on the above cultural and morphological characters and biochemical characters given in table 8, the bacterium is identified as *Pseudomonas* sp.

4.5.3. Identification of bacterial isolates by MTCC, IMTECH, Chandigarh

Two bacterial isolates, MCN-3 and PFC-4 were sent to Microbial Type Culture Collections (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh for further confirmation of identification. The isolate MCN-3 was identified as *Geobacillus kaustophilus* with the accession No. MTCC 8517 and the isolate PFC-4 was identified as *Pseudomonas fluorescens* with the accession No. MTCC 8518.

4.6. EXTRACTION OF BIOSURFACTANTS FROM THE SELECTED BACTERIA

Eight promising BS isolates were subjected to centrifugation followed by evaporation and acetone precipitation for the extraction of BS. The results are presented in Table 9. Among the BS bacteria, KFS1 (*Pseudomonas* sp.) from *Eucalyptus* plantations, Peechi recorded maximum BS production (7.95 g /l) followed by MCN-3 (*Geobacillus kaustophilus*) (6.45 g /l) and DTSC3- *Pseudomonas* sp. (6.20 g/l) isolated from chlorpyriphos treated plot and mancozeb treated vegetable plot respectively. A moderate amount of BS (4.60 g/l– 5.90 g/l) was produced by the isolates viz., PFC-4 -*Pseudomonas fluorescens* (*Eucalyptus* plantations), KFN2 -*Pseudomonas* sp. (*Eucalyptus* plantations) and DTSC5 - *Pseudomonas* sp. (mancozeb treated vegetable plot). A minimum BS was recorded by the isolates KCC-2 (*Pseudomonas* sp.) (2.90 g/l) and MCC-2 *Pseudomonas* sp. (3.50 g/l) from Kunnathuvalappil ayurveda nursing home and chlorpyriphos treated plot respectively.

4.7. ESTIMATION OF HYDROCARBON EMULSIFYING ACTIVITY OF BIOSURFACTANT BY XYLENE EMULSIFICATION ASSAY

To study the bioactivity of the BS extracted from the bacterial isolates, xylene emulsification activity was determined using the standard procedure as described earlier. Light absorbance values were read spectrometrically at 610 nm at 1 h and 24 h later and the results were presented in Table 10 (Fig. 3).

A high emulsification activity was observed at 1h and at 24 h in all the eight isolates tested. After one hour, the isolate KFS1 showed highest emulsifying activity (0.910) followed by the isolates MCN-3 (*Geobacillus kaustophilus*) and KCC-2 -*Pseudomonas* sp. (0.875 and 0.790 respectively). After 24 h, all the isolates were recorded increased emulsification activity where the isolate

Table 9. Quantity of biosurfactants produced by different bacterial isolates

Sl.No.	Bacterial isolates	Weight of biosurfactant produced (g/l) *
1.	KCC-2 (*Pseudomonas* sp)	2.90
2.	MCC-2 (*Pseudomonas* sp)	3.50
3.	MCN-3 (*Geobacillus kaustophilus*)	6.45
4.	KFS1 (*Pseudomonas* sp.)	7.95
5.	KFN2 (*Pseudomonas* sp.)	4.70
6.	PFC-4 (*Pseudomonas fluorescens*)	4.60
7.	DTSC3 (*Pseudomonas* sp)	6.20
8.	DTSC5 (*Pseudomonas* sp)	5.90

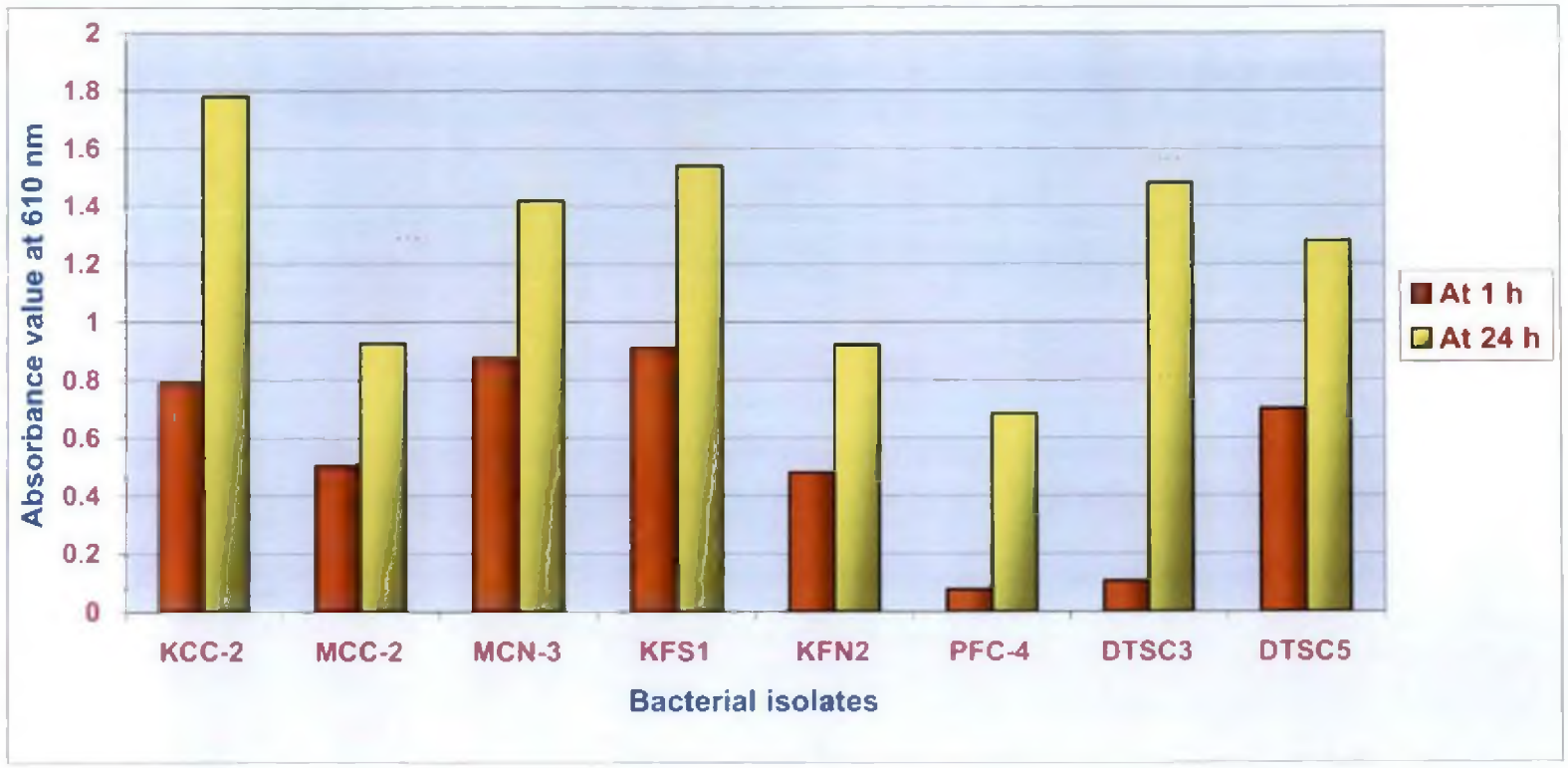
*Mean of three replications

Table 10. Xylene emulsification activity of the biosurfactants

Sl.No.	Bacterial isolates	Absorbance value at 610nm.*	
		At 1h.	At 24h.
1.	KCC-2 (*Pseudomonas* sp)	0.790	1.780
2.	MCC-2 (*Pseudomonas* sp)	0.504	0.925
3.	MCN-3 (*Geobacillus kaustophilus*)	0.875	1.420
4.	KFS1 (*Pseudomonas* sp.)	0.910	1.540
5.	KFN2 (*Pseudomonas* sp.)	0.480	0.920
6.	PFC-4 (*Pseudomonas fluorescens*)	0.076	0.684
7.	DTSC3 (*Pseudomonas* sp)	0.105	1.480
8.	DTSC5 (*Pseudomonas* sp)	0.700	1.280

*Mean of three replications

Fig. 3. Xylene emulsification activity of biosurfactant of selected bacterial isolates



KCC-2 -*Pseudomonas* sp.
MCC-2 - *Pseudomonas* sp.
MCN-3 -*Geobacillus kaustophilus*
KFS1 - *Pseudomonas* sp.
KFN2 - *Pseudomonas* sp.
PFC-4 - *Pseudomonas fluorescens*
DTSC3- *Pseudomonas* sp.
DTSC5 - *Pseudomonas* sp.

KCC-2 gave maximum emulsification (1.780) followed by the isolates KFS1 *Pseudomonas* sp. and DTSC3 *Pseudomonas* sp. (1.540 and 1.480 respectively). The other isolates also recorded a good order of emulsification at 1 h, which slightly increased after 24 h.

4.8. ESTIMATION OF SURFACE TENSION OF LIQUIDS BY THE ACTIVITY OF BIOSURFACTANT BY DROP WEIGHT METHOD

The surface tension (ST) values of distilled water, glycerol (10^{-1} dilution), cyclohexane and methoxy ethanol monomethyl ether without the addition of BS were recorded as 0.073, 0.091, 0.046 and 0.048 Nm^{-1}respectively. When these liquids were treated with biosurfactants extracted from the eight isolates, the surface tension values were lowered (Table 11, Fig. 4). When the BS of DTSC5 *Pseudomonas* sp. isolate was treated with the liquids viz., water, glycerol (10^{-1} diliution), cyclohexane and methoxy ethanol monomethyl ether, ST values were reduced to a minimum level of 0.021, 0.023, 0.022 and 0.010 Nm^{-1} respectively. BS produced by the isolate, PFC-4 *Pseudomonas fluorescens* (*Eucalyptus* plantations, Peechi) also lowered the ST values of all the four liquids tested to a minimum level of 0.032, 0.035, 0.030 and 0.032 respectively. Biosurfactant produced by the other six isolates also reduced the ST values of all the liquids tested. Surface tension lowering of the liquids indicated the increased bioactivity of the BS produced by the isolates.

4.9. EFFECT OF NUTRITIONAL AND CULTURAL CONDITIONS ON BIOSURFACTANT PRODUCTION AND EMULSIFICATION ACTIVITY

4.9.1. Effect of sugar sources on the BS production and emulsification activity

Four sugar sources viz., sucrose, maltose, mannitol and glucose were tested to study their effect on BS production and emulsification activity and the results are furnished in Table 12.

Table 11. Reduction of surface tension of liquid by the activity of biosurfactants

Sl. No.	Liquid + biosurfactant	Surface Tension (N/m) *
1.	Distilled water control	0.073
2.	Distilled water +*Pseudomonas* sp. (KCC-2)	0.045
3.	Distilled water + *Pseudomonas* sp. (MCC-2)	0.052
4.	Distilled water + *Geobacillus kaustophilus* (MCN-3)	0.051
5.	Distilled water + *Pseudomonas* sp. (KFS1)	0.051
6.	Distilled water + *Pseudomonas* sp. (KFN2)	0.045
7.	Distilled water + *Pseudomonas fluorescens* (PFC-4)	0.032
8.	Distilled water + *Pseudomonas* sp. (DTSC3)	0.039
9.	Distilled water + *Pseudomonas* sp. (DTSC5)	0.021
1.	Glycerol control	0.091
2.	Glycerol + *Pseudomonas* sp. (KCC-2)	0.075
3.	Glycerol + *Pseudomonas* sp. (MCC-2)	0.076
4.	Glycerol+ *Geobacillus kaustophilus* (MCN-3)	0.042
5.	Glycerol + *Pseudomonas* sp. (KFS1)	0.083
6.	Glycerol + *Pseudomonas* sp. (KFN2)	0.063
7.	Glycerol + *Pseudomonas fluorescens* (PFC-4)	0.035
8.	Glycerol + *Pseudomonas* sp. (DTSC3)	0.069
9.	Glycerol + *Pseudomonas* sp. (DTSC5)	0.023

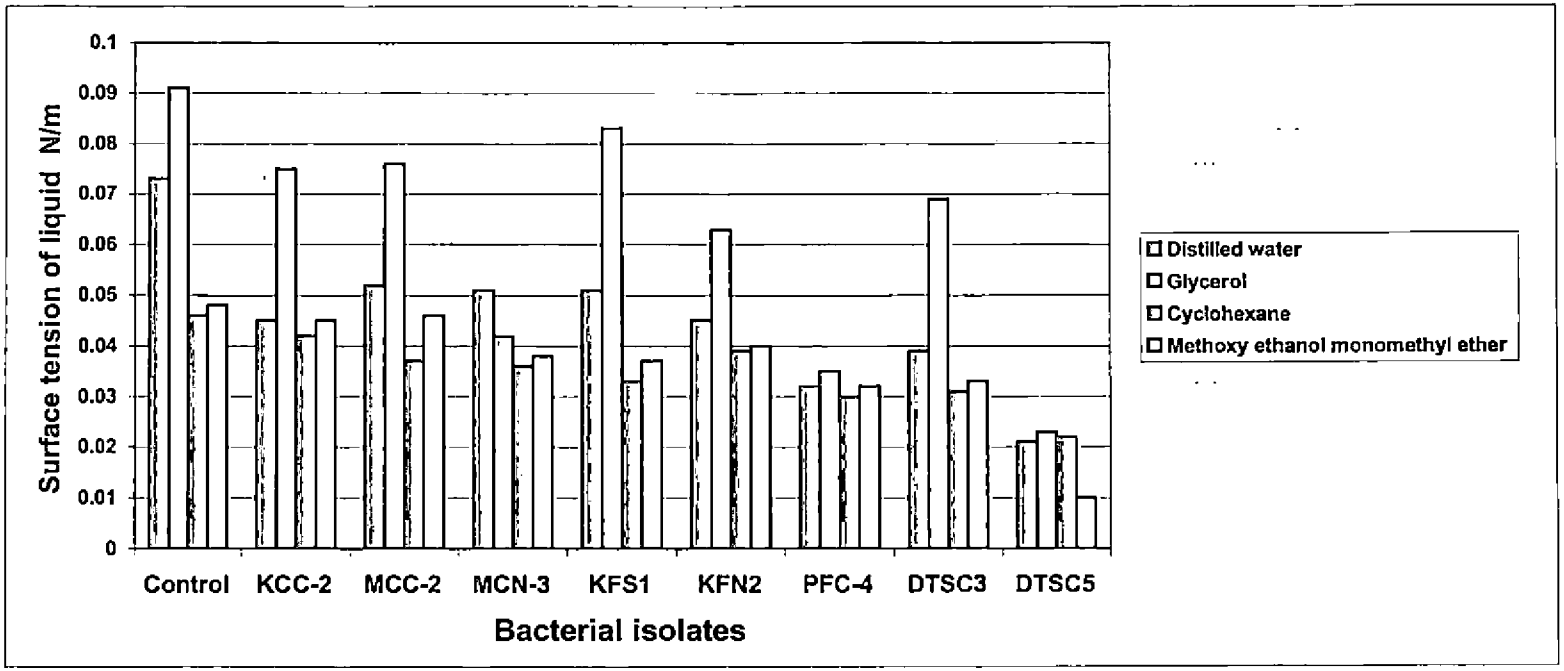
(Continued)

Sl. No.	Liquid +biosurfactant	Surface Tension (N/m)*
1.	Cyclohexane control	0.046
2.	Cyclohexane+*Pseudomonas* sp. (KCC-2)	0.042
3.	Cyclohexane + *Pseudomonas* sp. (MCC-2)	0.037
4.	Cyclohexane + *Geobacillus kaustophilus* (MCN-3)	0.036
5.	Cyclohexane + *Pseudomonas* sp. (KFS1)	0.033
6.	Cyclohexane + *Pseudomonas* sp. (KFN2)	0.039
7.	Cyclohexane + *Pseudomonas fluorescens* (PFC-4)	0.030
8.	Cyclohexane+*Pseudomonas* sp. (DTSC3)	0.031
9.	Cyclohexane + *Pseudomonas* sp. (DTSC5)	0.022
1.	Methoxy ethanol monomethyl ether control	0.048
2.	Methoxy ethanol monomethyl ether + *Pseudomonas* sp. (KCC-2)	0.045
3.	Methoxy ethanol monomethyl ether + *Pseudomonas* sp. (MCC-2)	0.046
4.	Methoxy ethanol monomethyl ether + *Geobacillus kaustophilus* (MCN-3)	0.038
5.	Methoxy ethanol monomethyl ether + *Pseudomonas* sp. (KFS1)	0.037
6.	Methoxy ethanol monomethyl ether + *Pseudomonas* sp. (KFN2)	0.040
7.	Methoxy ethanol monomethyl ether + *Pseudomonas fluorescens* (PFC-4)	0.032
8.	Methoxy ethanol monomethyl ether + *Pseudomonas* sp. (DTSC3)	0.033
9.	Methoxy ethanol monomethyl ether + *Pseudomonas* sp. (DTSC5)	0.010

Mean of three replications

* Expressed as Newtons per meter

Fig. 4. Reduction of surface tension of liquids by the activity of biosurfactants



KCC-2 - *Pseudomonas* sp.
MCC-2 - *Pseudomonas* sp.
MCN-3 -*Geobacillus kaustophilus*
KFS1 - *Pseudomonas* sp.
KFN2 - *Pseudomonas* sp.
PFC-4 - *Pseudomonas fluorescens*
DTSC3- *Pseudomonas* sp.
DTSC5 - *Pseudomonas* sp.

Maximum BS production (9.60 g /l) was recorded by the isolate MCN-3 *Geobacillus kaustophilus* (chlorpyriphos treated plot), when mannitol was used as sugar source. A significantly lesser BS production was noticed when other sugars viz., sucrose, maltose and glucose used as carbon source by this bacteria (Fig.5). Xylene emulsification assay was maximum (0.467) when sucrose was used as carbon source, which was on par with glucose (0.444). Minimum emulsification value was noticed when maltose and mannitol used as sugar source. In the case of KFS1 isolate (*Eucalyptus* plantations), maximum biosurfactant (5.9 g /l) was noticed when glucose was substituted in the media, which was on par with sucrose and mannitol. Maximum emulsification activity was obtained when glucose used as sugar source (Fig.6). In DTSC3 isolate - *Pseudomonas* sp., BS production and emulsification activity was maximum when glucose used as sugar source. In KFN2 isolate *Pseudomonas* sp. mannitol recorded maximum BS production and emulsification activity.

4.9.2. Effect of hydrocarbon sources on BS production and emulsification activity

Four hydrocarbon sources viz., cyclohexane, xylene, neem oil and kerosene were tested to study their effect on BS production and emulsification activity and results presented in Table 13.

In the case of MCN-3 isolate, BS production was maximum (8.5 g /land 8.4 g/l) when media were substituted with xylene or neem oil. Maximum emulsification activity was recorded due to cyclohexane (0.755), followed by neem oil and xylene substituted media. The isolate KFS1 recorded maximum BS production in xylene substituted media (7.2 g/l) followed by cyclohexane (6.07 g/l). But BS of the KFS1 isolate recorded maximum emulsification (0.758) when cyclohexane used as hydrocarbon source that was followed by neem oil and xylene. DTSC3 isolate recorded maximum BS production by neem oil substituted media (8.00 g/l) followed by xylene (7.6 g/l). Emulsification activity

Table 12. Effect of sugar sources on the production and emulsification activity of biosurfactant

Sl.No.	Bacterial isolates	Sugar sources							
		Sucrose		Maltose		Mannitol		Glucose	
		BS production(g/l)	Absorbance value at 610nm	BS production(g/l)	Absorbance value at 610nm	BS production(g/l)	Absorbance value at 610nm	BS production (g/l)	Absorbance value at 610nm
1	MCN-3	2.68	0.467	1.44	0.235	9.60	0.290	3.03	0.444
2	KFS1	5.80	0.409	2.90	0.277	5.20	0.217	5.90	0.826
3	DTSC3	2.57	0.292	2.87	0.209	2.80	0.332	3.57	0.850
4	KFN2	3.50	0.257	3.17	0.218	6.03	0.577	4.70	0.246

Each treatment replicated thrice

$CD_{(0.05)}$ (BS production) - 0.695

$CD_{(0.05)}$ (Emulsification activity) - 0.136

Fig. 5. Effect of sugars on the production of biosurfactant

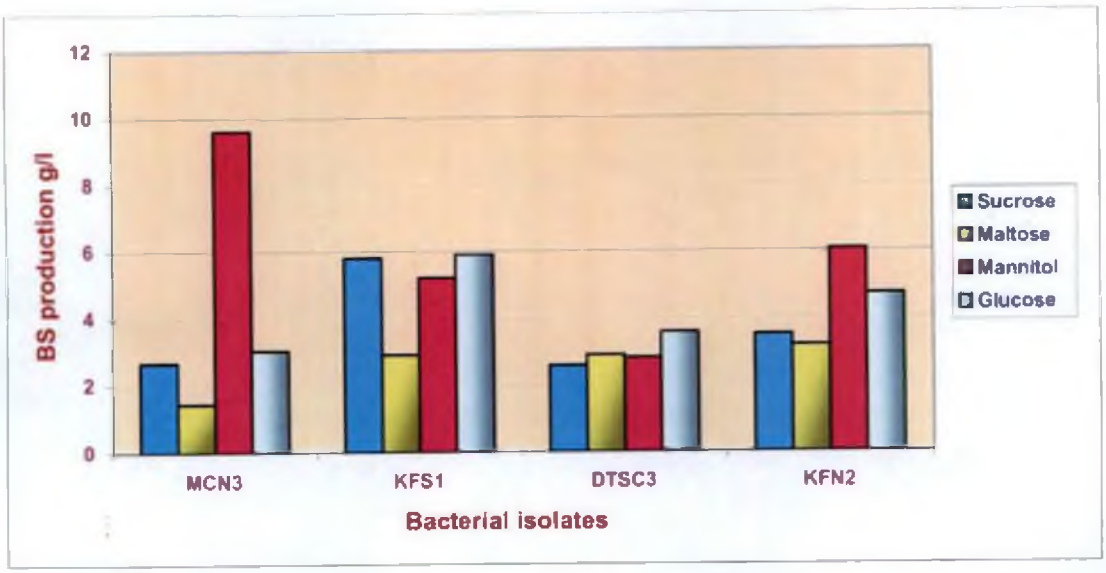
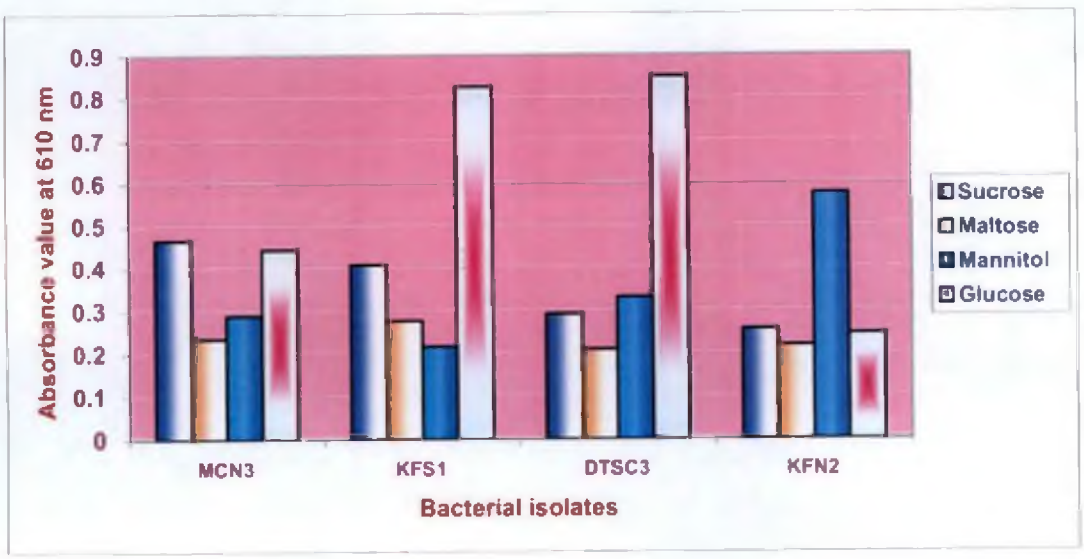


Fig. 6. Effect of sugars on the emulsifying activity of biosurfactant



MCN-3	**-*Geobacillus kaustophilus***	**DTSC3**	**- *Pseudomonas* sp.**
KFS1	**- *Pseudomonas* sp.**	**KFN2**	**- *Pseudomonas* sp.**

Table 13 Effect of hydrocarbon sources on the production and emulsification activity of biosurfactant

Sl.No.	Bacterial isolates	Hydrocarbon sources							
		Cyclohexane		Xylene		Neem oil		Kerosene	
		BS production(g/l)	Absorbance value at 610nm	BS production(g/l)	Absorbance value at 610nm	BS production(g/l)	Absorbance value at 610nm	BS production (g/l)	Absorbance value at 610nm
1	MCN-3	2.80	0.755	8.50	0.518	8.40	0.530	4.37	0.118
2	KFS1	6.07	0.758	7.20	0.419	3.20	0.531	3.30	0.191
3	DTSC3	3.80	0.221	7.60	0.313	8.00	0.388	5.47	0.169
4	KFN2	5.00	0.322	5.60	0.303	6.00	0.325	3.30	0.184

Each treatment replicated thrice

$CD_{(0.05)}$ (BS production) - 1.10

$CD_{(0.05)}$ (Emulsification activity) - 0.097

Fig. 7. Effect of hydrocarbons on the production of biosurfactant

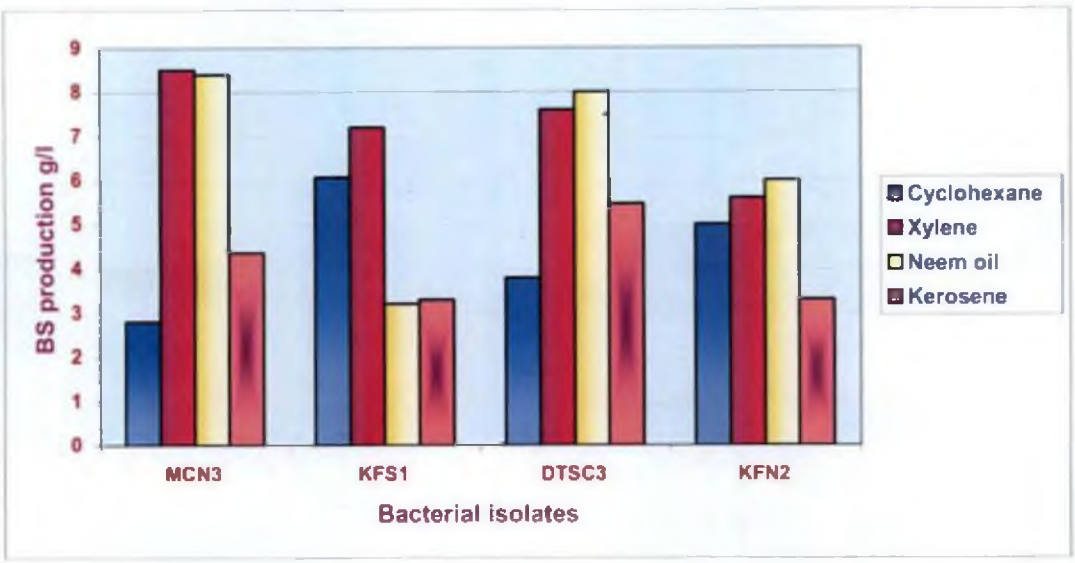
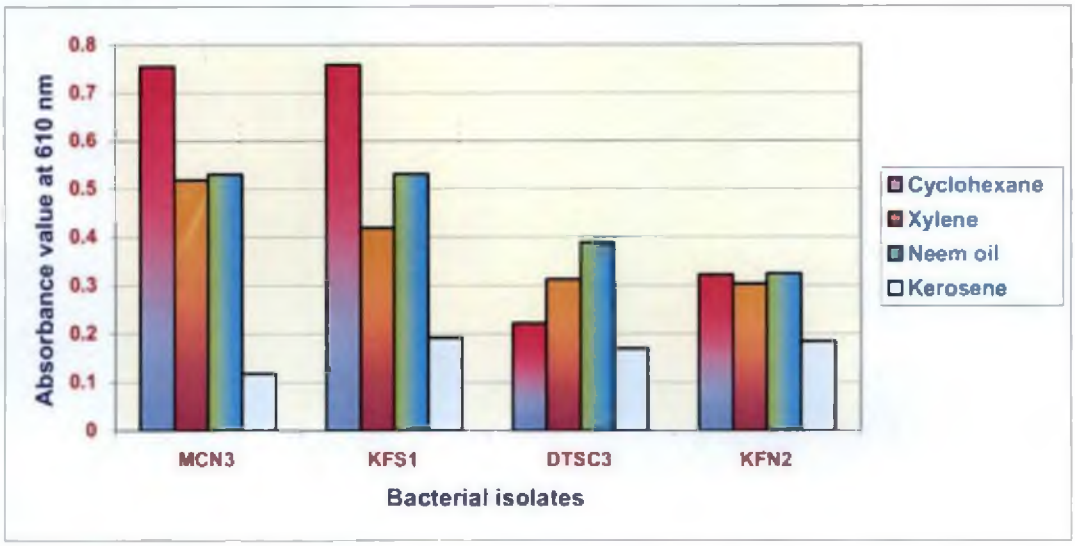


Fig.8. Effect of hydrocarbons on the emulsifying activity of biosurfactant



MCN-3	**-*Geobacillus kaustophilus***	**DTSC3**	**- *Pseudomonas* sp.**
KFS1	**- *Pseudomonas* sp.**	**KFN2**	**- *Pseudomonas* sp.**

was maximum due to neem oil and xylene substituted media (0.388 and 0.313 respectively). The isolate KFN2 recorded maximum BS production by neem oil (6.00 g/l), which was on par with xylene (5.60 g/l) and cyclohexane (5.00 g/l) substituted media. Except kerosene, the other three hydrocarbons viz., xylene, neem oil and cyclohexane recorded a fairer emulsification activity (0.325, 0.322 and 0.303 respectively) by the KFN2 isolate (Fig. 7 and Fig. 8).

4.9.3. Effect of pH on BS production and emulsification activity

The BS production and emulsification activity were determined at different pH (5.0, 6.0, 7.0 and 8.0) and the results are furnished (Table 14, Fig. 9 and Fig. 10). The favourable pH for maximum BS production in the case of MCN-3 isolate was pH 7.0 and was on par with pH 6.0 and pH 8.0, where as the emulsification activity was maximum at pH 8.0 (0.778) and on par with at pH 7.0 (0.723). KFS1 isolate recorded maximum BS at pH 8.0 (6.36 g/l), which was on par with pH 7.0 (6.23 g/l) and pH 6.0 (5.80 g/l). The emulsification was found to be maximum at pH 7.0 (0.504) and was on par with pH 8.0 (0.499).

In the case of DTSC3 and KFN2 isolates, the favourable pH for BS production ranged from 6.0 – 8.0 (4.73 g/l – 5.67 g/l and 7.50 g/l – 7.90 g/l respectively), but emulsification value was obtained at pH 7.0 – 8.0 (0.408 – 0.436 and 0.492 – 0.498 respectively) by the BS of these two isolates.

4.9.4. Effect of temperature on BS production and emulsification activity

Optimum temperature for the BS production and emulsification activity of the bacterial isolates was determined by incubating the culture broth at 20^0C, 30^0C and 40^0C and the results are presented in Table 15 (Fig. 11 and Fig. 12).

From the table it is observed that, the isolates MCN-3 and KFS1 recorded maximum BS production at 30^0C (4.93 g/l and 5.03 g/l respectively) and its

Table 14 Effect of pH on the production and emulsification activity of biosurfactant

Sl.No.	Bacterial isolates	Different pH							
		5pH		6 pH		7 pH		8 pH	
		BS production (g/l)	Absorbance value at 610nm	BS production (g/l)	Absorbance value at 610nm	BS production (g/l)	Absorbance value at 610nm	BS production (g/l)	Absorbance value at 610nm
1	MCN-3	2.63	0.285	3.43	0.680	3.90	0.723	3.40	0.778
2	KFS1	5.13	0.383	5.80	0.385	6.23	0.504	6.36	0.499
3	DTSC3	2.17	0.252	4.73	0.354	5.67	0.408	5.20	0.436
4	KFN2	6.47	0.430	7.50	0.407	7.60	0.492	7.90	0.498

Each treatment replicated thrice

$CD_{(0.05)}$ (BS production) - 1.10

$CD_{(0.05)}$ (Emulsification activity) - 0.062

Fig. 9. Effect of pH on the production of biosurfactant

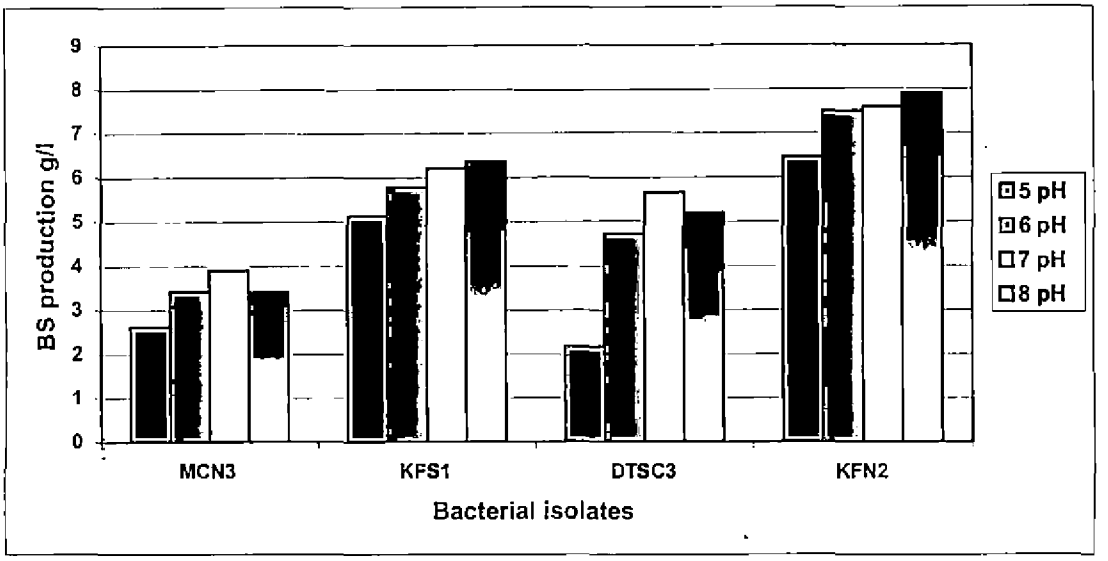
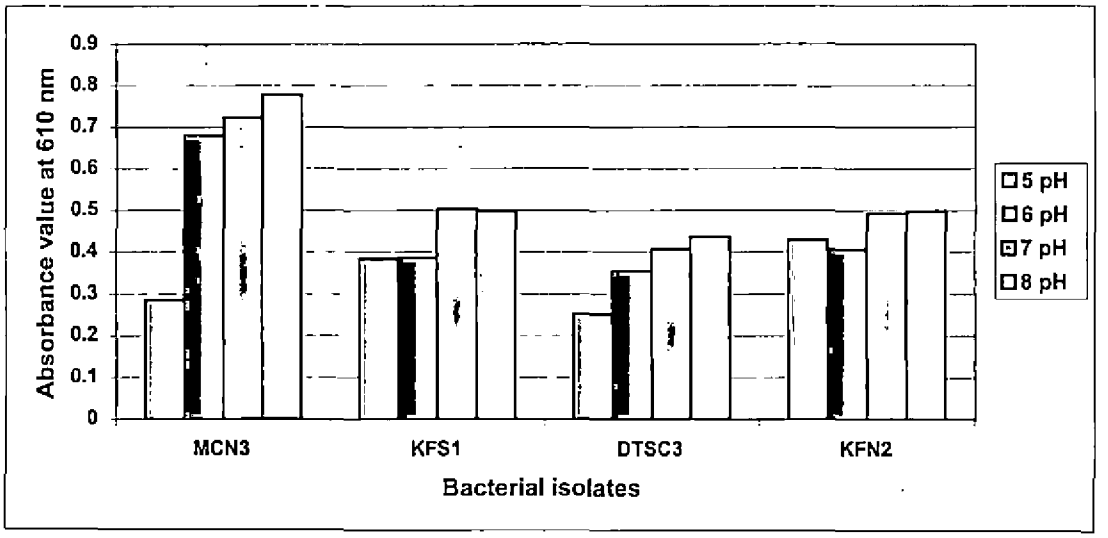


Fig. 10. Effect of pH on the emulsifying activity of biosurfactant



MCN-3	-*Geobacillus kaustophilus*	DTSC3	- *Pseudomonas* sp.
KFS1	- *Pseudomonas* sp.	KFN2	- *Pseudomonas* sp.

Table 15. Effect of temperature on the production and emulsification activity of biosurfactant

Sl.No.	Bacterial isolates	Different temperatures					
		20 ^{0}C		30 ^{0}C		40 ^{0}C	
		BS production(g/l)	Absorbance value at 610nm	BS production(g/l)	Absorbance value at 610nm	BS production(g/l)	Absorbance value at 610nm
1	MCN-3	3.15	0.282	4.93	0.341	4.00	0.276
2	KFS1	2.95	0.253	5.03	0.405	0.33	0.030
3	DTSC3	3.10	0.227	2.30	0.250	0.50	0.039
4	KFN2	3.07	0.243	5.83	0.351	0.18	0.017

Each treatment replicated thrice

$CD_{(0.05)}$ (BS production) - 0.653

$CD_{(0.05)}$ (Emulsification activity) - 0.080

Fig. 11. Effect of temperature on the production of biosurfactant

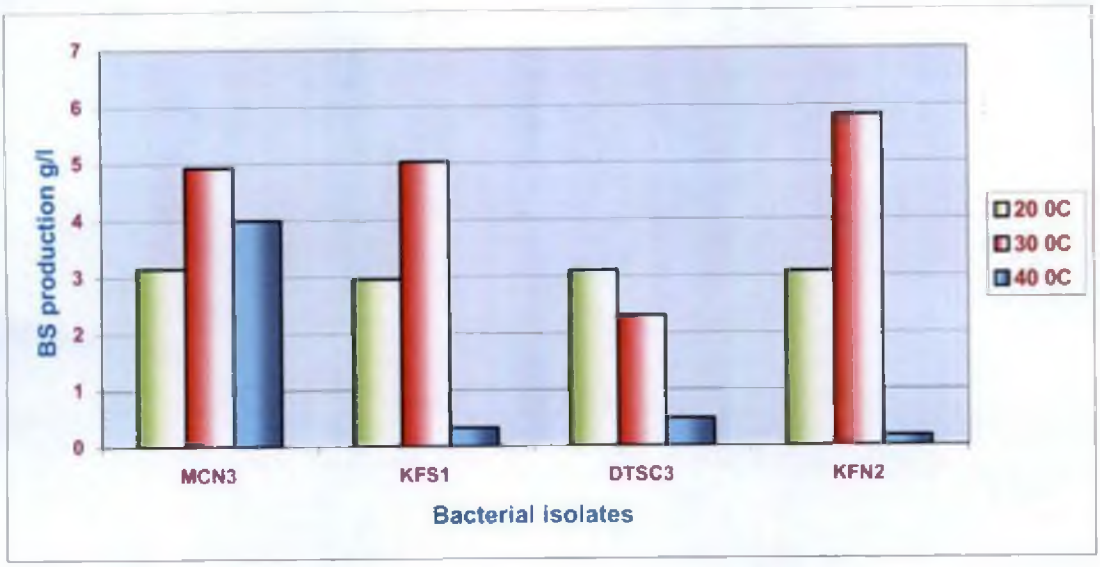
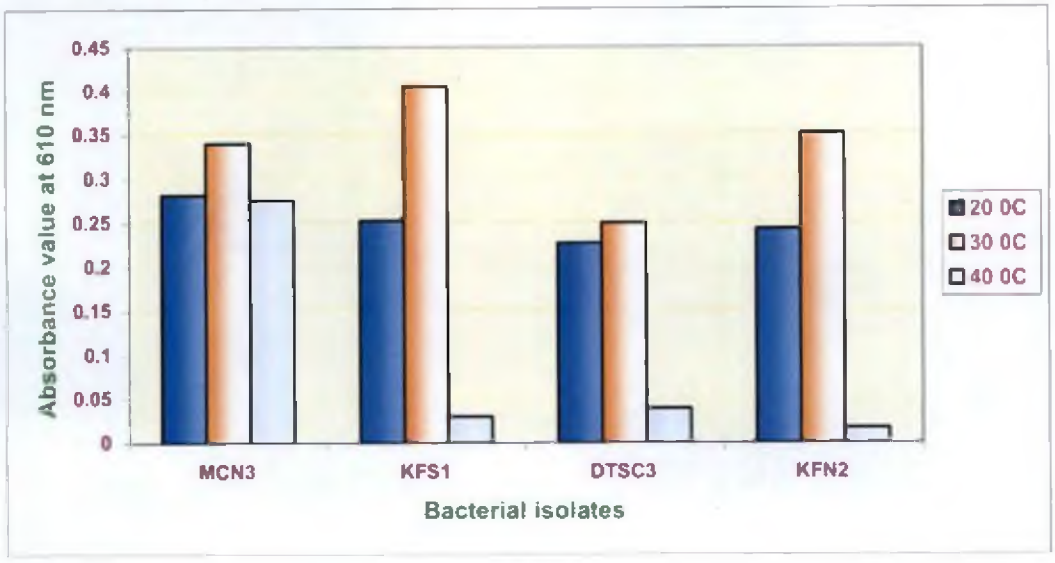


Fig. 12. Effect of temperature on the emulsifying activity of biosurfactant



MCN-3	**-*Geobacillus kaustophilus***	**DTSC3**	**- *Pseudomonas* sp.**
KFS1	**- *Pseudomonas* sp.**	**KFN2**	**- *Pseudomonas* sp.**

emulsification activity was also maximum at the same temperature. DTSC3 isolate produced maximum BS (3.10 g/l) at 20^0C and the emulsification activity at 30^0C (0.351). The isolate KFN2 recorded maximum BS production at 30 ^{0}C and its highest emulsification activity was also observed at the same temperature. Optimum temperature for BS production and emulsification varied with the isolates.

Optimum nutritional and cultural conditions for the BS production and emulsification activity of promising BS producing bacteria are summarized and presented in Table 16.

4.10. STUDIES ON THE DEGRADATION OF PESTICIDES BY BIOSURFACTANT BACTERIA

4.10.1. Degradation of chlorpyriphos

Residue analysis of chlorpyriphos was carried out as per the standard protocol described in section 3.11.1. and the results are presented in Table 17. The concentration of chlorpyriphos in the control (soil sample without bacteria), after the application of insecticide was calculated as 55.84 µg /g soil. However, in bacteria treated soil samples concentration of chemical was found to be declined at 40 days after application (DAA). The highest reduction in concentration (16.03 µg /g) was recorded by KFS1 isolate treated soil samples followed by MCN-3 and DTSC3 treated soils. The chromatogram of standard chlorpyriphos, MCN-3, KFS1 and DTSC3 samples were furnished in Fig. 13,14,15 and 16).

Per cent degradation of chlorpyriphos over control was calculated. The BS bacteria, KFS1 (*Eucalyptus* plantations, Peechi) recorded the highest per cent degradation of chlorpyriphos (71.29per cent) followed by the isolate MCN-3, from

Table 16. Optimum nutritional and cultural conditions for the biosurfactant production and emulsification activity of promising BS producing bacteria

Bacterial isolate	Sugar source		Hydrocarbon source		Optimum pH		Optimum temperature	
	BS production	Xylene emulsification	BS production	Xylene emulsification	BS production	Xylene emulsification	BS production	Xylene emulsification
MCN-3 *(Geobacillus kaustophilus)*	Mannitol	Sucrose	Xylene	Cyclohexane	pH 7.0	pH 8.0	30^0C	30^0C
KFS1 *(Pseudomonas* sp.)	Glucose	Glucose	Xylene	Cyclohexane	pH 8.0	pH 7.0	30^0C	30^0C
DTSC3 *(Pseudomonas* sp.)	Glucose	Glucose	Neem oil	Neem oil	pH 7 .0	pH 8.0	20^0C	30^0C
KFN2 *(Pseudomonas* sp.)	Mannitol	Mannitol	Neem oil	Neem oil	pH 8.0	pH 8.0	30^0C	30^0C

Table 17. Effect of BS bacteria on the degradation of chlorpyriphos (40 days after application)

Bacterial isolate	Source of the isolate	Concentration of the chemical (μ g /g soil)*	Per cent degradation over control
MCN-3 *(Geobacillus kaustophilus)*	Chlorpyriphos treated plot	46.42	16.87
KFS1 (*Pseudomonas* sp.)	*Eucalyptus* plantations, Peechi	16.03	71.29
DTSC3 (*Pseudomonas* sp.)	Mancozeb treated vegetable plot	55.17	1.20
Control	--	55.84	--

* Mean of three replications

Fig. 13. Chromatogram of standard chlorpyriphos 0.05ppm

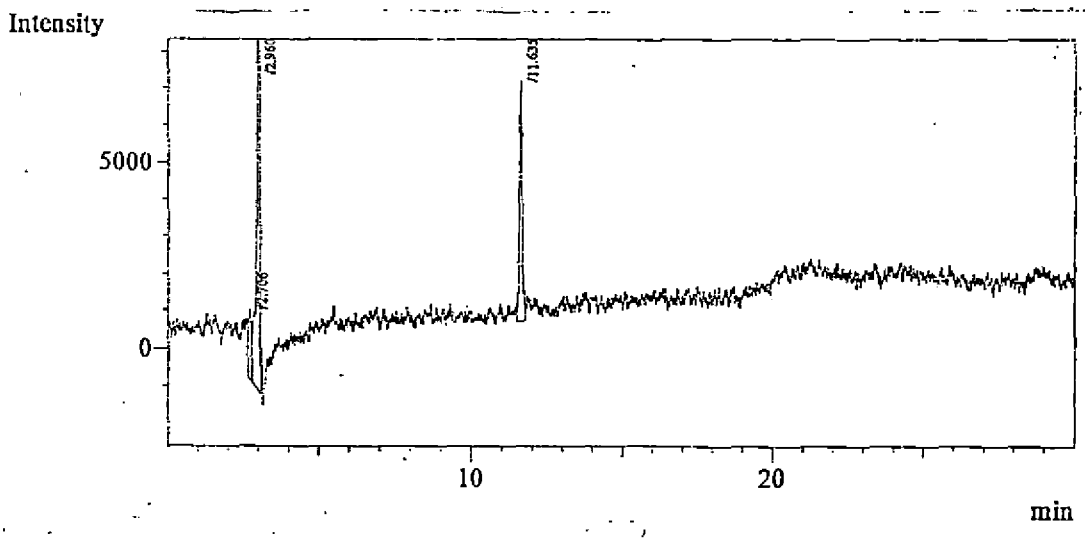


Fig. 14. Chromatogram of chlorpyriphos in MCN-3 sample at 40DAA

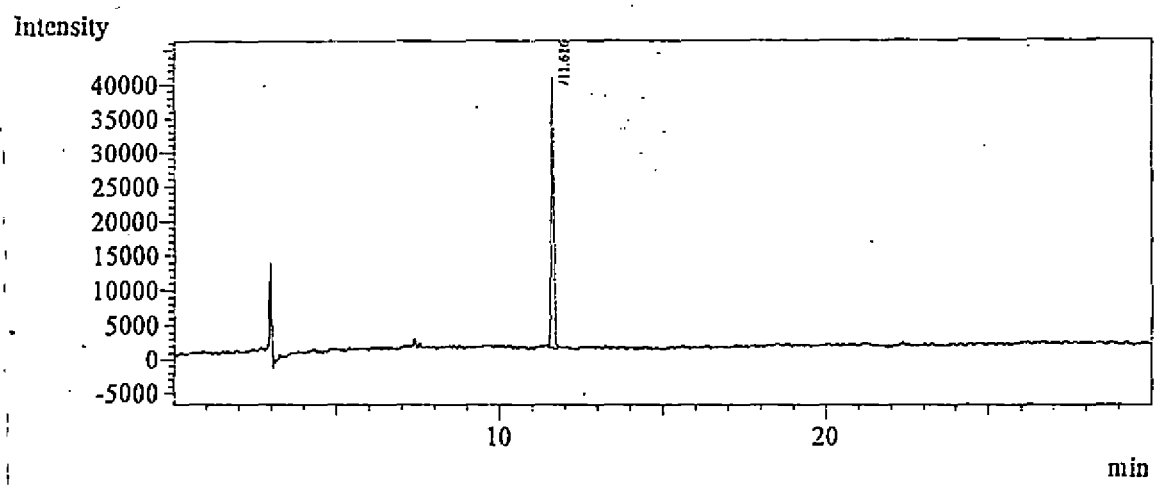


Fig. 15. Chromatogram of chlorpyriphos in KFS1 sample at 40DAA

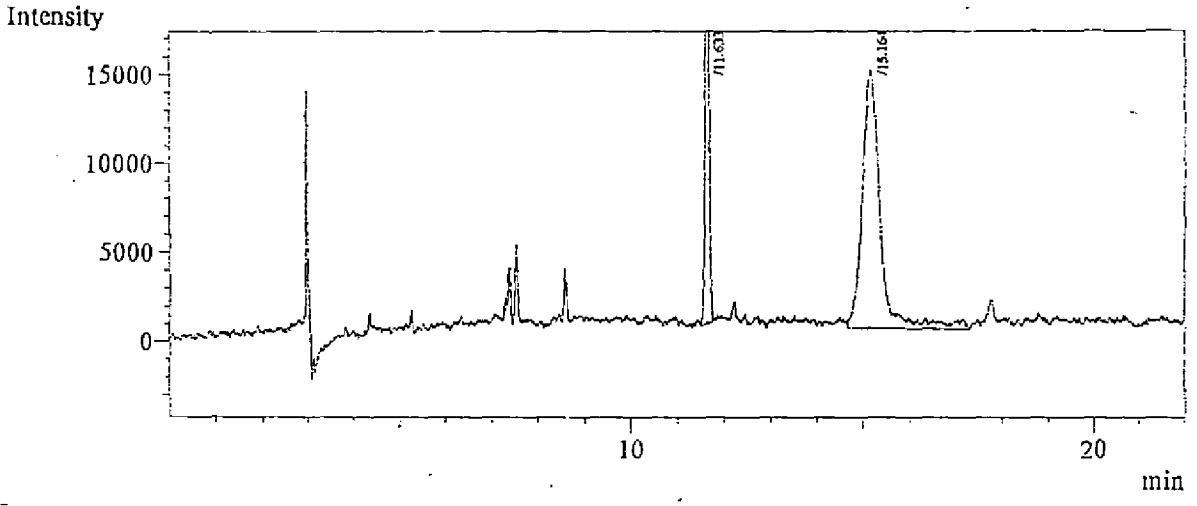
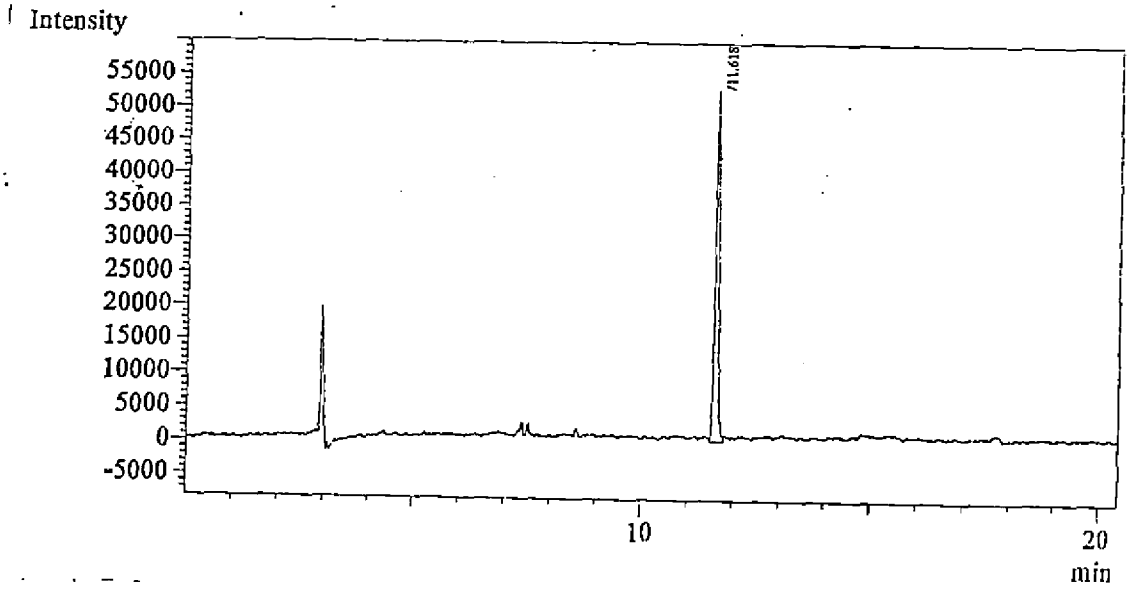


Fig. 16. Chromatogram of chlorpyriphos in DTSC3 sample at 40DAA



chlorpyriphos treated soil sample (16.87per cent). Minimum degradation (1.2 per cent) was noticed with DTSC3 from mancozeb treated soil samples.

4.10.2. Degradation of mancozeb

Residue analysis of mancozeb was carried out at intervals of 5, 10 and 40 days after application (DAA) as per the standard protocol described in 3.11.2. and the results are furnished in Table 18.

The concentration of mancozeb in the control (soil sample without bacteria) was calculated as 0.738 µg /g soil at 5 DAA where as, in bacteria treated samples a gradual reduction in concentration was noticed at 5, 10 and 40 DAA of chemical.

Among the three bacterial isolates treated samples, DTSC3 - *Pseudomonas* sp. treated soils recorded a highest reduction in concentration compared to the other isolates treated samples and controls. At 40 DAA, DTSC3 treatment recorded a highest reduction in concentration (0.478 µg /g) compared to other treatments and control. Per cent degradation of mancozeb were calculated. Out of the three bacterial treated soils and one control, the DTSC3 (isolated from mancozeb treated vegetable plot) treated sample registered maximum degradation (28.44 per cent) followed by MCN-3 -*Geobacillus kaustophilus* (23.95 per cent) at 40 DAA of mancozeb.

Table 18. Effect of BS bacteria on degradation of mancozeb

Bacterial isolate	Source of the isolate	Concentration * of the chemical (μ g /g soil) (5DAA)	Per cent reduction over control	Concentration* of the chemical (μ g /g soil) (10DAA)	Per cent reduction over control	Concentration * of the chemical (μ g /g soil) (40DAA)	Per cent reduction over control
MCN-3 *(Geobacillus kaustophilus)*	Chlorpyriphos treated plot	0.681	7.72	0.568	19.08	0.507	23.95
KFS1 (*Pseudomonas* sp.)	*Eucalyptus* plantations, Peechi	0.707	4.20	0.634	9.69	0.574	14.07
DTSC3 (*Pseudomonas* sp.)	Mancozeb treated vegetable plot	0.677	8.26	0.541	22.93	0.478	28.44
CONTROL	--	0.738	--	0.702	--	0.668	--

* Mean of three replications DAA - Days after application

4.11. *In vitro* ANTIMICROBIAL ACTIVITY OF SELECTED BIOSURFACTANT BACTERIA AGAINST SOIL BORNE PATHOGENS AND BIOCONTROL AGENTS

4.11.1. *In vitro* effect of the biosurfactant producing bacteria against selected soil borne pathogens

4.11.1.1. Pythium aphanidermatum

The per cent inhibition of *Pythium aphanidermatum* by BS bacteria are presented in Table 19. Among the eight BS producing bacterial isolates tested against the pathogen *Pythium aphanidermatum*, DTSC3 isolate (*Pseudomonas* sp.) was found most effective against *P. aphanidermatum*, recording an inhibition of 54.4 per cent by streaking on one side and 55.5 per cent by streaking on both sides followed by MCN-3 (*Geobacillus kaustophilus*) (52.2. per cent and 50.0 per cent respectively) and DTSC5 (47.4 per cent and 49.4 per cent respectively) (Plate IV.A).

4.11.1.2. Phytophthora capsici

The effect of eight selected BS producing bacteria against *P.capsici*, revealed that except two isolates (MCC2 and KCC-2) the other six isolates recorded good percent inhibition to *P.capsici*. (Table 20). The isolates viz., DTSC3 and DTSC5 gave maximum per cent inhibition by both methods and at the point of contact of bacteria, mycelial lysis of the pathogen was observed at four days after incubation (Plate IV.B). Between the two methods adopted, streaking on both sides was found as the most effective method compared to streaking on one side.

Table 19. *In vitro* evaluation of biosurfactant producing bacteria against *Pythium aphanidermatum*

Sl. No.	Bacterial isolates	Growth of *Pythium aphanidermatum* Days after incubation (Diameter in mm)*					Per cent inhibition
			1	2	3	4	
1.	KCC-2	I	20.0	61.6	86.0	88.0	2.2
		II	17.5	30.0	76.0	76.0	15.5
2.	MCC-2	I	20.6	31.0	51.0	73.3	18.5
		II	15.0	25.0	64.0	64.0	28.8
3.	MCN-3	I	19.6	31.0	43.0	43.0	52.2
		II	20.0	32.0	45.0	45.0	50.0
4.	KFS1	I	20.0	32.3	45.6	51.6	42.6
		II	17.5	31.5	50.0	50.0	44.4
5.	KFN2	I	19.6	59.3	75.0	84.0	6.6
		II	22.5	30.0	70.5	70.5	21.6
6.	PFC-4	I	19.3	54.0	60.0	76.3	15.2
		II	17.5	27.5	65.0	65.0	27.7
7.	DTSC3	I	20.3	31.3	41.0	41.0	54.4
		II	15.0	20.0	40.0	40.0	55.5
8.	DTSC5	I	20.3	31	47.3	47.3	47.4
		II	16.5	22.0	45.5	45.5	49.4
9	Control (*P. aphanidermatum*)		21.0	64.6	87.0	90.0	--

I – Streaking on one side II – Streaking on both side

* Mean of three replications

Table 20. *In vitro* evaluation of biosurfactant producing bacteria against *Phytophthora capsici*

Sl. No.	Bacterial isolates		Growth of *Phytophthora capsici* Days after incubation (Diameter in mm)*									Per cent inhibition
			1	2	3	4	5	6	7	8	9	
1.	KCC-2	I	12.3	26.6	37.0	53.0	61.0	69.3	75.3	78.6	86.0	4.4
		II	12.0	30.0	39.0	53.0	65.0	80.0	85.0	87.0	90.0	0
2.	MCC-2	I	14.6	27.0	36.3	40.6	52.0	61.3	68.6	72.0	84.0	6.6
		II	15.0	24.5	29.0	33.0	51.0	52.0	67.0	76.0	88.0	2.2
3.	MCN-3	I	15.3	26.6	41.0	50.6	50.6	50.6	50.6	50.6	50.6	43.7
		II	16.0	31.0	47.0	49.0	55.0	55.0	55.0	55.0	55.0	38.8
4.	KFS1	I	16.0	27.6	34.6	39.0	45.0	45.0	45.0	45.0	45.0	50.0
		II	17.0	29.0	37.0	39.0	40.0	40.0	40.0	40.0	40.0	55.5
5.	KFN2	I	16.0	28.0	37.6	43.6	46.6	48.3	48.3	48.3	48.3	46.3
		II	15.0	29.0	35.0	40.0	42.0	42.5	54.0	54.0	54.0	40.0
6.	PFC-4	I	16.0	27.0	36.3	43.0	52.3	60.3	66.0	68.6	71.3	20.7
		II	18.0	30.0	36.0	44.0	50.0	62.0	63.6	65.0	65.0	27.7
7.	DTSC3	I	15.0	25.6	29.6	32.0	36.6	39.3	39.3	39.3	39.3	56.3
		II	12.0	30.0	39.0	39.0	39.0	39.0	39.0	39.0	39.0	56.6
8.	DTSC5	I	15	25.6	37.0	39.3	40.3	40.3	40.3	40.3	40.3	55.2
		II	15.0	30.0	37.0	37.0	37.0	37.0	37.0	37.0	37.0	56.6
9.	Control (*Phytophthora capsici*)		16.0	30.0	41.0	53.0	63.3	71.3	83.6	86.0	90.0	--

I – Streaking on one side II– Streaking on both side * Mean of three replications

4.11.1.3. Rhizoctonia solani

When *R. solani* was tested with the eight BS producing bacterial isolates, MCN-3 from chlorpyriphos treated soil gave maximum per cent inhibition followed by DTSC3, PFC-4 and DTSC5 isolates (Table 21). The isolates KCC-2, KFS1 and KFN2 did not record any noticeable inhibition towards the growth of the pathogen. Presence of sclerotia was observed in all the tested plates (Plate IV C). The two methods adopted were found to be effective in testing the antimicrobial activity of bacterial isolates.

Summing up of the results (Fig. 17), it is observed that among the eight bacterial isolates DTSC5, DTSC3 and MCN-3 were found to be most effective against all the three soil borne pathogens as they exhibited maximum per cent inhibition.

4.11.2. Studies on the compatibility of biosurfactant producing bacteria with biocontrol agents

4.11.2.1. Pseudomonas fluorescens

Compatibility of eight biosurfactant producing bacteria with standard culture of *P. fluorescens* was tested by cross streaking and by point inoculation methods. In cross streaking method, no lysis was observed at the junction of biosurfactant producing bacteria and *P. fluorescens*.

However in case of point inoculation method an inhibition zone of 7.8% and 8.9% were noticed with MCN-3 -*Geobacillus kaustophilus* and KCC-2-*Pseudomonas* sp. isolates (Table 22) (Plate V A).

Table 21. *In vitro* evaluation of biosurfactant producing bacteria against *Rhizoctonia solani*

Sl. No.	Bacterial isolates	Growth of *Rhizoctonia solani* Days after incubation (Diameter in mm)*						Per cent inhibition	Presence of sclerotia
			1	2	3	4	5		
1.	KCC-2	I	17.3	45.3	69.0	80.6	90.0	0	+
		II	15.0	30.3	56.4	79.0	80.6	10.4	
2.	MCC-2	I	16.6	42.6	60.0	67.6	81.6	9.3	+
		II	13.0	26.6	39.5	59.3	59.3	34.1	
3.	MCN-3	I	16.6	41.3	51.6	51.6	51.6	42.6	+
		II	12.9	28.3	29.5	30.3	30.3	66.3	
4.	KFS1	I	16.3	41.3	50.6	69.3	84.5	6.1	+
		II	14.5	38.3	66.3	73.0	73.0	18.8	
5.	KFN2	I	17.6	45.6	61.3	81.0	90.0	0	+
		II	15.0	36.0	57.0	69.0	69.0	23.3	
6.	PFC-4	I	17.3	42.6	52.6	53.0	53.5	40.5	+
		II	12.2	32.3	35.0	41.3	41.3	54.1	
7.	DTSC3	I	17.0	42.6	51.6	56.6	57.0	36.6	+
		II	12.5	18.6	20.0	31.0	31.0	65.5	
8.	DTSC5	I	16.6	41.6	51.6	52.6	53.0	41.1	
		II	12.8	28.6	30.0	35.0	35.0	61.1	+
9.	Control (*R. solani*)		17.6	45.6	73.6	81.8	90.0	--	+

I– Streaking on one side II– Streaking on both side + Present

*Mean of three replications

PLATE – IV

Antimicrobial activity of BS bacteria against soil borne pathogens

A. *Pythium aphanidermatum*

(1) KFS1
(2) MCC-2

Control

MCN-3

B. *Phytophthora capsici*

DTS C3

KFS 1

MCN-3

Control

C. *Rhizoctonia solani*

DTS C3

MCN-3

MCC-2

Control

Table 22. Compatibility of selected biosurfactant producing bacteria with *Pseudomonas fluorescens* (Point inoculation method)

Sl. No.	Bacterial isolates	Standard biocontrol agent	Diameter of inhibition zone (mm)*	Per cent inhibition
1	KCC-2	*P. fluorescens*	7	7.8
2	MCC-2		--	0
3	MCN-3		8	8.9
4	KFS1		--	0
5	KFN2		--	0
6	PFC-4		--	0
7	DTSC3		--	0
8	DTSC5		--	0

* Mean of three replications

4.11.2.2. Trichoderma harzianum

In the compatibility study of *T. harzianum* with BS bacteria, there was no inhibition observed by the fungus and in all test plates the fungus covered well as that of control. The bacterial culture from the test plate was restreaked to test the antagonistic activity of fungus against the bacteria and it was found that all isolates of bacteria retained its viability (Table 23) (Plate V B).

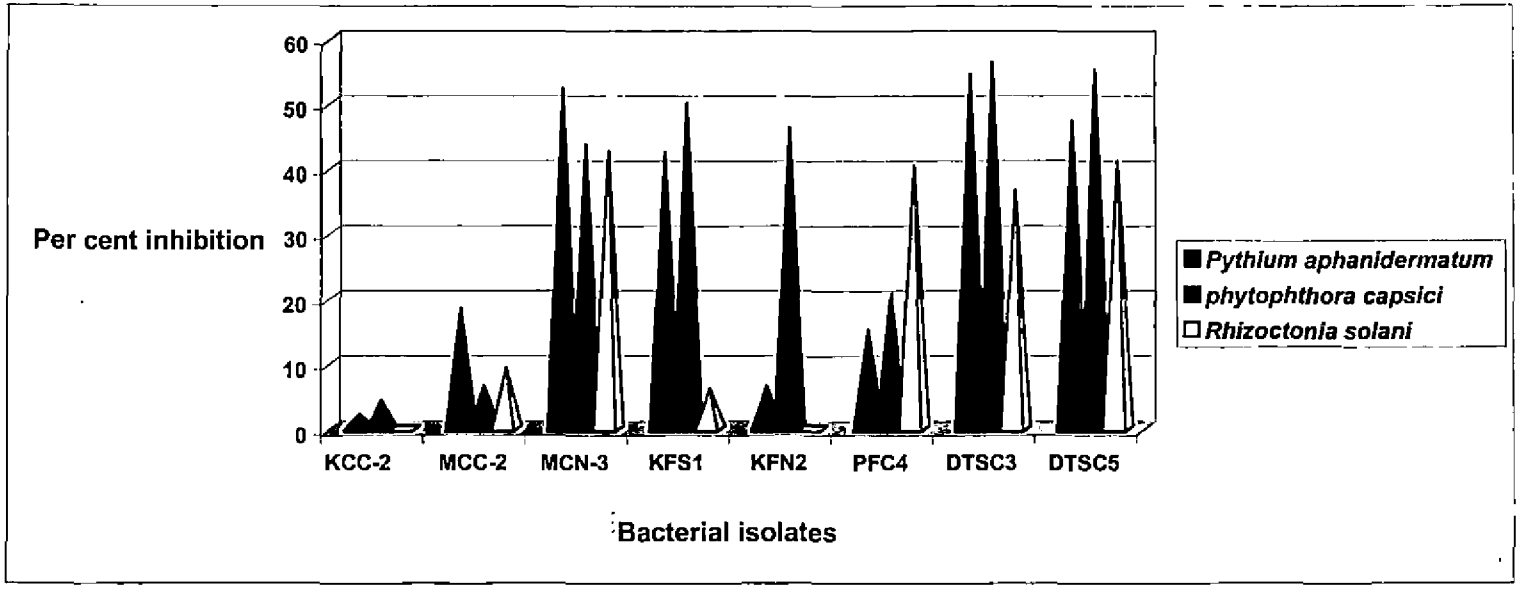
4.11.2.3. Trichoderma viride

As in the case of *T. harzianum* all the eight biosurfactant producing bacteria were found to be compatible with *T.viride* (Table 24). It is also showed that all bacterial isolates retained its viability when restreaked from the test plates (Plate V C).

4.12. GROWTH PROMOTION EFFECT OF SELECTED BIOSURFACTANT PRODUCING BACTERIA

The plant growth promotion effect of selected BS bacteria was tested on cowpea and sorghum seeds. In cowpea seeds, germination per cent ranged from 90 to 100 and in sorghum seeds 80 to 100. Significant difference recorded in plumule length of cowpea seeds with respect to different isolates. Maximum plumule length 7.22 cm was observed with MCN-3, which followed by PFC-4 5.88 cm where as, KCC-2 and KFN2 had no effect. In case of radicle length, maximum with MCN-3 followed by PFC-4. KCC-2, KFN2 and DTSC3 had no effect as compared to control. In sorghum seeds, maximum plumule length was noticed with MCC-2 isolate that was on par with MCN-3, PFC-4, KFN2 and KFS1. Radicle length as maximum due to treatment of MCN-3, that was on par with KFS1, PFC-4 and MCC-2. KCC-2 had no effect on plumule and radicle length compared to control.

Fig. 17. Antimicrobial activity of selected biosurfactant bacteria against soil borne pathogens



KCC-2 ***Pseudomonas*** **sp.**

MCC-2 ***Pseudomonas*** **sp.**

MCN-3 ***Geobacillus kaustophilus***

KFS1 ***Pseudomonas*** **sp.**

KFN2 - ***Pseudomonas*** **sp.**

PFC-4 - ***Pseudomonas fluorescens***

DTSC3- ***Pseudomonas*** **sp.**

DTSC5 - ***Pseudomonas*** **sp.**

Table 23. Compatibility studies of selected biosurfactant producing bacteria with *Trichoderma harzianum*

Sl. No.	Bacterial isolates	Growth of *Trichoderma harzianum* Days after incubation (Diameter in mm)*					Percent inhibition
			1	2	3	4	
1.	KCC-2	I	24.6	58.0	81.6	90.0	0
		II	21.0	63.0	90.0	90.0	
2.	MCC-2	I	22.0	58.3	83.0	90.0	0
		II	20.0	64.0	90.0	90.0	
3.	MCN-3	I	26.3	62.6	88,6	90.0	0
		II	21.0	73.0	90.0	90.0	
4.	KFS1	I	23.6	62.0	86.0	90.0	0
		II	21.0	68.0	90.0	90.0	
5.	KFN2	I	29.6	61.6	87.6	90.0	0
		II	22.0	71.0	90.0	90.0	
6.	PFC-4	I	23.3	57.6	68.6	90.0	0
		II	21.0	63.0	90.0	90.0	
7.	DTSC3	I	28.3	67.0	88.3	90.0	0
		II	20.0	65.0	90.0	90.0	
8.	DTSC5	I	24.3	57.3	81.3	90.0	0
		II	20.0	76.0	90.0	90.0	
9.	Control (*Trichoderma harzianum)*		29.6	67.5	89.0	90.0	--

I – Streaking on one side II– Streaking on both side

* Mean of three replications

Table 24. Compatibility studies of selected biosurfactant bacteria with *Trichoderma viride*

Sl. No.	Bacterial isolates	Growth of *Trichoderma viride* Days after incubation (Diameter in mm)*					Percent inhibition
			1	2	3	4	
1.	KCC-2	I	20.3	60.3	82.3	90.0	0
		II	24.2	60.0	90.0	90.0	
2.	MCC-2	I	18.3	60.0	85.6	90.0	0
		II	23.0	62.0	90.0	90.0	
3.	MCN-3	I	20.3	64.6	85.3	90.0	0
		II	22.0	56.0	90.0	90.0	
4.	KFS1	I	22.3	69.3	87.6	90.0	0
		II	21.0	68.0	90.0	90.0	
5.	KFN2	I	20.6	61.6	80.0	90.0	0
		II	22.0	65.0	90.0	90.0	
6.	PFC-4	I	20.6	67.0	87.6	90.0	0
		II	20.5	55.0	90.0	90.0	
7.	DTSC3	I	20.3	53.6	73.6	90.0	0
		II	21.6	59.0	90.0	90.0	
8.	DTSC5	I	20.0	59.6	80.0	90.0	0
		II	22.5	65.0	90.0	90.0	
9.	Control (*Trichoderma viride*)		20.6	70.0	87.6	90.0	--

I – Streaking on one side II – Streaking on both side

* Mean of three replications

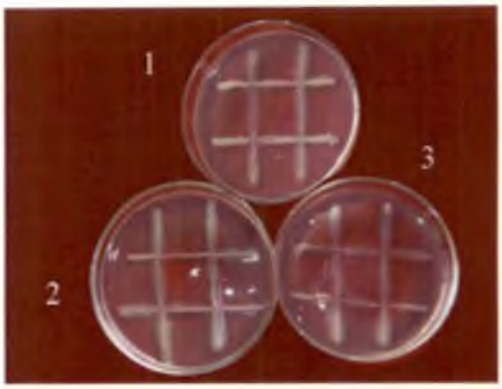
PLATE – V

Compatibility of BS bacteria with bioagents

A. *Pseudomonas fluorescens*

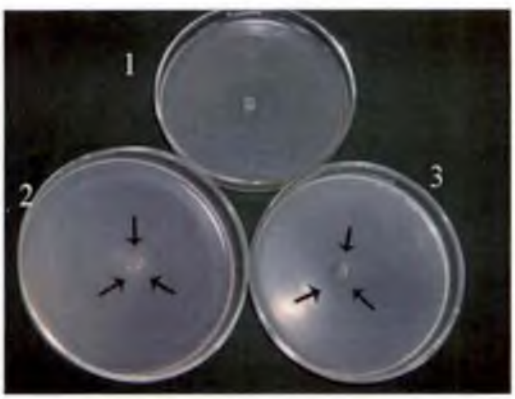
Cross streaking method

(1) MCN- 3
(2) PFC- 4



(1) DTSC 3
(2) DTSC 5
(3) KCC - 2

Point inoculation method

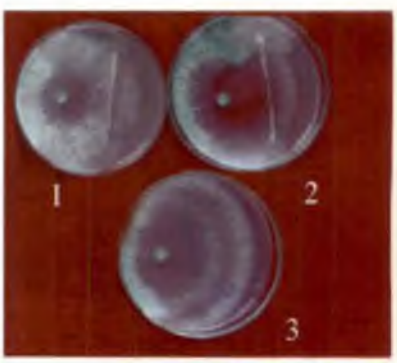


(1) DTSC 3
(2) KCC - 2
(3) MCN- 3

B. *Trichoderma harzianum*

(1) KFS 1
(2) Control

C. *Trichoderma viride*



(1) KCC- 2
(2) KFS 1
(3) Control

From the table (Table 25) it is observed that all bacterial isolates had effect on germination of both cowpea and sorghum seeds as compared to control.

Summing up the findings so far on the characters of selected BS bacteria presented in Table 26, it is revealed that characters were varied with isolates. However, isolates viz., DTSC3 - *Pseudomonas* sp., KFS1 - *Pseudomonas* sp. and MCN-3 - *Geobacillus kaustophilus* are the most superior cultures with respect to all characters studied.

Table 25. Growth promotion effect of selected biosurfactant bacteria

Sl.No.	Bacterial isolates	Cowpea seeds			Sorghum seeds		
		Germination per cent	Radicle length (cm)	Plumule length (cm)	Germination per cent	Radicle length (cm)	Plumule length (cm)
1	KCC-2	100	2.560^{e}	4.190^{c}	90	1.745^{bc}	1.136^{c}
2	MCC-2	100	4.640^{bc}	5.800^{b}	100	2.972^{a}	1.705^{a}
3	MCN-3	100	6.020^{a}	7.220^{a}	100	3.178^{a}	1.696^{a}
4	KFS1	100	4.210^{cd}	5.400^{bc}	100	3.123^{a}	1.550^{a}
5	KFN2	90	2.860^{e}	4.400^{bc}	100	2.662^{b}	1.617^{a}
6	PFC-4	100	5.720^{ab}	5.880^{b}	100	3.056^{a}	1.655^{a}
7	DTSC3	100	3.080^{de}	5.780^{b}	100	2.845^{ab}	1.466^{ab}
8	DTSC5	100	4.340^{cd}	5.670^{b}	90	2.662^{b}	1.492^{ab}
9	Control	90	4.280^{cd}	4.760^{bc}	80	2.453^{b}	1.222^{bc}

In each column figures followed by same letter do not differ significantly according to DMRT

Table 26. Important characters of the BS producing bacterial isolates from selected soils of Kerala

Bacterial isolate	BS production (g/l)	Area of dispersion of bacteria		Biosurfactant activity		per cent degradation of pesticides		Antimicrobial action (Per cent inhibition)		Compatibility	
		Drop collapse (mm^2)	Xylene spray (mm^2)	Xylene emulsification (OD at 610nm)	ST of water (N/m)	Mancozeb (µg/g)	Chlorpyriphos (µg/g)	*Pythium*	*Phytophthora*	*P. fluorescens*	*T. harzianum* and *T. viride*
KCC-2 (*Pseudomonas* sp.)	2.90	170.0	289.4	1.780	0.045	Not studied	Not studied	2.2	4.4	Less compatible	No inhibition
MCC-2 (*Pseudomonas* sp.)	3.50	251.6	834.3	0.925	0.052	- do -	- do -	18.5	6.6	Compatible	- do -
MCN-3 (*Geobacillus kaustophilus*)	6.45	310.0	642.1	1.420	0.051	24.07	16.87	52.2	43.7	Less compatible	- do -
KFS1 (*Pseudomonas* sp.)	7.95	276.6	615.4	1.540	0.051	13.99	71.29	42.6	20.7	Compatible	- do -
KFN2 (*Pseudomonas* sp.)	4.70	333.0	961.6	0.920	0.045	Not studied	Not studied	6.6	50	Compatible	- do -
PFC-4 (*Pseudomonas fluorescens*)	4.60	170.7	289.4	0.684	0.032	- do -	- do -	15.2	46.3	Compatible	- do -
DTSC3 (*Pseudomonas* sp.)	6.20	324.6	783.9	1.480	0.039	28.35	1.20	54.4	56.3	Compatible	- do -
DTSC5 (*Pseudomonas* sp.)	5.90	218.5	854.9	1.280	0.021	Not studied	Not studied	47.4	55.2	Compatible	- do -

5. DISCUSSION

Human activities have severe impact on the environment, especially during the last two centuries with the advent of industrial activities. Nowadays there is a general awareness about the detrimental affects of certain products and subproducts from industrial processes and that their release and disposal to the environment should be controlled or even prevented. Several techniques like incineration and chemical treatments are available to clean contaminated soils. Recently bioremediation has gained acceptance as an alternative for pollutant removal. Availability of microorganisms with catabolic potential to degrade the target pollutants has now become indispensable.

Surface active compounds produced by microorganisms are potentially useful in agriculture, especially in various formulations of herbicides and pesticides, which help the active ingredients uniformly dispensed in the aqueous solutions. For the bioremediation of hydrocarbon-contaminated soils, *in situ* production of biosurfactants by seeding the native bacterial cultures has been successfully practiced.

Perusal of the literature revealed scanty information about the biosurfactant producing bacteria from soils of Kerala. Thus the present work will be useful to study the potentiality of BS bacteria from selected soils of Kerala, its antimicrobial activity on pathogens as well as on beneficial microbes and their effect on the degradation of pesticides.

5.1. ISOLATION OF TOTAL HETEROTROPHIC BACTERIA FROM SELECTED SOIL SAMPLES

Soil is the storehouse of microorganisms and soil microbes display biodiversity of a high magnitude. It should be possible to isolate the potential BS producing microorganisms from soil environment. By selective enrichment of soils such microorganisms can be preferentially stimulated and isolated (Purushothaman, 2002). So, in the present study attempts were made to isolate total heterotrophic bacteria from different soil samples. Soils were collected from forest area, automobile spillovers, and ayurvedic nursing homes and from permanent areas of herbicidal and pesticidal trials. Selective enrichments were given to the soils by adding hydrocarbons like neem oil and crude oil.

In the present study, population of heterotrophic bacteria in hydrocarbon contaminated soil samples were differed significantly. Among the different soil samples tried, bacterial population was significantly higher in neem oil and crude oil enriched soil samples when compared to control (without enrichment). Soils from chlorpyriphos treated plot and herbicide treated plot- Location 2 were recorded a maximum bacterial population of 124.0×10^7 and 79.67×10^7 cfu/g respectively, due to neem oil enrichment, where as the same soil samples recorded bacterial population of 100.0×10^7 and 74.67×10^7cfu/g due to crude oil enrichment. This suggested that, selective enrichment preferentially stimulated heterotrophic bacterial population. However, Maruti service station and Indian oil petroleum bunk were recorded a moderate bacterial population (53.33×10^7 and 42.67×10^7 cfu/g respectively) in non-enriched samples and did not show any drastic increase due to continuous spill over of petroleum and oil in these areas, samples were already enriched with hydrocarbons, so additional enrichment did not show any influence on the bacterial population.

Numerous reports (Soli and Bens, 1973; Miget, 1973; Kator, 1973; Traxler, 1973) show that increase in microbial population occurs due to the addition of hydrocarbons. The addition of hydrocarbons will enrich primarily for microorganisms that utilize hydrocarbons and secondarily for microorganisms capable of utilizing metabolites of hydrocarbon degrading microorganisms. Such enrichment results in increased number of hydrocarbon utilizing microorganisms and associated secondary colonizers, which in turn results in an overall increase of microbial population present within the ecosystem. The effect of petroleum hydrocarbons on the size of microbial populations will depend upon the chemical composition of the contaminating hydrocarbons and on the species of microorganisms present within the microbial community of the particular ecosystem (Bartha and Atlas, 1977).

5.2. SCREENING OF BACTERIAL ISOLATES FOR SURFACTANT PRODUCTION ACTIVITY

5.2.1. Drop collapse technique

After the isolation of heterotrophic bacteria, drop collapse assay was carried out to screen BS bacteria. Among the 92 bacteria screened, 36 bacteria have shown positive reaction to drop collapse assay. Here, the drops of fermentation broth containing BS bacteria soon collapsed and spread on neem oil coated plates. Among the 36 isolates, the isolate KFN2 (*Eucalyptus* plantation) gave the maximum area of dispersion (333 mm^2) followed by DTSC3 (324.60 mm^2) from mancozeb treated vegetable plot and MCN-3 (310 mm^2) from chlorpyriphos treated plot. A moderate area of dispersion of bacterial culture drops were showed by other isolates viz., KFS1 (276.60 mm^2), MCC-2 (251.6 mm^2), DTSC5 (218.5 mm^2), PFC-4 (170.66 mm^2) and KCC-2 (170mm^2).

Drop collapse assay deviced by Jain *et al.* (1991), is the widely accepted screening method for surfactant producing microorganisms. Drops of cell suspensions of surfactant producing colonies collapsed on an oily-coated surface. Colonies that did not produce, or produced very low concentration of surfactants remained stable. This established that the stability of drops was dependent on BS concentration and it had correlated with surface tension but not with emulsifying activity. Microbial colonies grown in the presence of hydrocarbons could be readily screened for surfactant production by this method.

In the present study neem oil was used for the induction of biosurfactants by the bacterial isolates. Syal and Ramamurthy (2003) reported vegetable oils containing unsaturated fatty acids, such as sunflower oil and neem oil are very good inducers of BS activity due to the presence of triglycerides which served as good substrates for growth and BS production. Neem oil being cheap and like other vegetable oils, it contains primarily of triglycerides of oleic, stearic, linoleic and palmitic acid (CSIR, 1985), it was used in the present study for the induction of biosurfactants. Thus BS production was in response to the hydrophobic fatty acid moiety of the neem oil.

5.2.2. Xylene spray method

This method was also adopted for screening BS producing bacteria. Among the 92 isolates screened, 24 isolates showed positive reaction by giving clear zones around the bacterial colonies on xylene sprayed plate. The isolate KFN2 from *Eucalyptus* plantations gave maximum area of clear zone (961.62 mm^2) followed by the DTSC5 isolate from mancozeb treated vegetable plot (854.86 mm^2) and MCC-2 from chlorpyriphos treated plot (834.26 mm^2). A moderate area of dispersion (289.38 mm^2–783.86 mm^2) was recorded by the isolates viz., KCC-2 (Kunnathuvalappil ayurveda nursing home), PFC-4 and KFS1 (*Eucalyptus* plantation), MCN-3

(chlorpyriphos treated plot) and DTSC3 (mancozeb treated vegetable plot) respectively.

Similar experiments were conducted by Kiyohara *et al.* (1982) and Burd and Ward (1996), where phenanthrene sprayed on pre- incubated bacterium showed a clear zone on nutrient agar plate. This result indicated that the bacterial colonies generated a transparent halo not only as a result of xylene degradation, but also by the solubilization of these hydrophobic compounds, mediated by biosurfactants released by the bacterial cells into the agar zone surrounding the colony.

Siegmund and Wagner (1991) reported an agar plate method for rapid screening of BS bacteria. Here, bacteria were streaked on mineral salt medium which produced a dark blue halo surrounding the colony after 24h of incubation, due to the production of rhamnolipid BS.

5.3. SELECTION OF BIOSURFACTANT PRODUCING BACTERIA

Based on the two screening tests like drop collapse and xylene spray method, eight BS bacteria that showed maximum area of dispersion were selected. The selected isolates were then characterized. Among the eight BS bacteria, except MCN-3 (gram positive rods) other seven isolates were observed as gram negative short rods. The isolates viz., MCN-3 and PFC-4 were authentically identified by Microbial Type Culture Collections, Institute of Microbial Technology, Chandigarh as *Geobacillus kaustophilus* and *Pseudomonas fluorescens* with their accession number MTCC 8517 and MTCC 8518 respectively. The other six isolates were identified as, *Pseudomonas* sp. (KCC-2); *Pseudomonas* sp. (KFS1); *Pseudomonas* sp. (KFN2); *Pseudomonas* sp (MCC-2); *Pseudomonas* sp, (DTSC3) and *Pseudomonas* sp, (DTSC5) respectively.

A search on literature revealed that, *Bacillus subtilis*, *B. cereus*, *Pseudomonas* spp., *Arthrobacter* spp., *Glucanobacter, Agrobacterium, Acinetobacter* spp. and *Mycobacterium* are notable bacterial species producing biosurfactants (Floodgate, 1984; Karanth *et al.*,1999; Purushothaman, 2002). Hauser and Karnovsky (1954) reported that several *Pseudomonas* sp. produced rhamnolipid BS, which is the most common type of biosurfactant. Christova *et al.* (2004) reported a new *Bacillus subtilis* 22BN strain on hydrocarbon degradation and rhamnolipid BS production.

In the present study, a new bacterium namely *Geobacillus kaustophilus* was isolated from chlorpyriphos treated plot, Mannarkad, Palakkad district, Kerala. Present investigation revealed that *G. kaustophilus* is a promising BS producing bacteria as evidenced by the screening tests, BS production, xylene emulsification assay and surface tension lowering effect. Antimicrobial activity against common soil borne pathogens and compatibility against certain bioagents are its added advantages. Similar to these findings, Nazina *et al.* (2001) also isolated certain hydrocarbon oxidizing *Bacillus* strains from high temperature oil fields of Kazakhstan which were later grouped into a new genera *Geobacillus*.

5.4. ESTIMATION OF BS PRODUCING BACTERIA IN SELECTED SOIL SAMPLES

Biosurfactant producing bacteria in selected soil samples were estimated from the total heterotrophic bacteria based on the standard screening test for BS bacteria. Soil samples from mancozeb treated vegetable plot (MSV-1) recorded maximum per cent BS bacteria of 45.4%. Samples from *Eucalyptus* plantations, Peechi (EFP-1) and forest lands of Wyanad (FSW-2) were scored 37.5per cent BS bacteria in both cases. Soil samples from Vydyaratnam Ayurveda Nursing Home did not record any BS bacteria out of the total heterotrophic bacteria. This suggested that, certain toxic compounds present in the drains of Ayurvedic nursing home might be detrimental to

the proliferation of BS bacteria. Bodour *et al.* (2003) reported that per cent BS bacterial population was dependent on soil conditions, with gram-positive biosurfactant producing isolates tending to be from heavy metal contaminated soils and gram-negative isolates tending to be from hydrocarbon contaminated soils.

5.5. EXTRACTION OF BIOSURFACTANT PRODUCED BY BACTERIAL ISOLATES

The ISM broth of eight selected BS bacteria viz., KCC-2, MCC-2, MCN-3, KFS1, KFN2, PFC-4, DTSC3 and DTSC5 were subjected to centrifugation followed by evaporation and acetone precipitation to extract the BS. These are the most widely used extraction procedures for BS (Cameotra, 1995). In the present study, KFS1 (*Eucalyptus* plantations) recorded maximum BS production (7.95 g/l), which was followed by the isolates MCN-3, *Geobacillus kaustophilus* (6.45 g /l) and DTSC 3, *Pseudomonas* sp (6.20 g /l). A moderate amount of BS (4.60– 5.90 g/l) produced by the isolates, PFC-4 *P. fluorescens* and KFN2 *Pseudomonas* sp. (*Eucalyptus* plantation); and DTSC5 *Pseudomonas* sp (mancozeb treated vegetable plot) respectively. A minimum BS was recorded by the isolates KCC-2 *Pseudomonas* sp. (2.90g/l) and MCC-2 *Pseudomonas* sp. (3.50g/l) from Kunnathuvalappil ayurveda nursing home and chlorpyriphos treated plot respectively.

Using standard procedures, BS were extracted from a variety of bacteria isolated from hydrocarbon or pesticide contaminated soil sites. Hauser and Karnovsky (1954) extracted glycolipid from several species of *Pseudomonas* and has been characterized as rhamnolipid. Suzuki *et al.* (1969) extracted BS, from the culture broth of *Arthrobacter paraffineus* when the cells were grown on hydrocarbon substrates, and the surfactants were characterized as trehalose lipids. Zhang and Miller (1992) reported extraction of BS from culture supernatant of bacteria using centrifugation and precipitation technique.

High amount of BS has been found in many bacteria. Yateem *et al* . (2002) isolated two BS producing *P.aeruginosa* strain, among this one strain produced two types of rhamnolipids with 98.4 g l^{-1} in nitrogen limited medium substituted with olive oil, where as the other strain produced only one type of rhamnolipid (5.9 g /l) in medium with crude oil. Deziel *et al.* (2003) extracted BS from the bacterium *P. aeruginosa* by inoculating it on mineral salt medium after mixing with one per cent $KHCO_3$ (pH 9). The whole content centrifuged and the supernatant acidified to pH 4.0 and the BS was extracted with ethyl acetate. Christova *et al.* (2004) reported that a new *Bacillus subtilis* strain which produced rhamnoliopid biosurfactant at high concentrations ranging from 1.5 - 2.0 g /l.

5.6. HYDROCARBON EMULSIFYING ACTIVITY OF BIOSURFACTANTS

The most frequently used indices for the performance of BS was the surface tension (ST) value and emulsification activity (Georgiou *et al.*1992).

In the present study, biosurfactant activity of the extracted BS from the eight isolates viz., KCC-2, MCN-3, MCC-2, KFS1, KFN2, PFC4, DTSC3 and DTSC5 were tested by xylene emulsification assay and by measuring the surface tension values of liquids.

5.6.1. Estimation of hydrocarbon emulsifying activity of biosurfactant by xylene emulsification assay

In the present study, the isolate KFS1 -*Pseudomonas* sp. showed highest emulsifying activity (0.910) after 1 h followed by the isolates MCN-3 *Geobacillus kaustophilus* and KCC-2 *Pseudomonas* sp. (0.875 and 0.790 respectively). After 24 h, all the isolates were recorded increased emulsification activity ranging from 0.684 to 1.780, among them the isolate KCC-2 gave maximum emulsification (1.780)

followed by the isolates KFS1 and DTSC3 *Pseudomonas* sp. (1.540 and 1.480 respectively). The other isolates also recorded a good order of emulsification at 1 h, which slightly increased after 24 h. This increase in absorbance at 24 h suggested that a higher absorbance might be due to a high level of dispersion of xylene in the buffer (Banat *et al* .1991).

Syal and Ramamurthy (2003) have conducted similar bioactivity studies of BS, by mixing Tris - HCl, culture broth, xylene and emulsification activity was determined by measuring the optical density of aqueous phase at 660nm. Barathi and Vasudevan (2001) isolated a strain *P. fluorescens* from petroleum hydrocorbon contaminated soil, and reported that the strain emulsified a number of aliphatic and aromatic hydrocarbons. Similar to these findings, Yateem *et al.* (2002) also found that the biosurfactant produced by *P. aeruginosa* strain are very effective in the emulsification of crude oil.

5.6.2. Estimation of surface tension of liquids by the activity of biosurfactant by drop weight method

In the present study, when ST value was measured, all the isolates showed a lower ST value in all liquids due to the presence of BS when compared to liquids alone (control). The isolate DTSC5 *Pseudomonas* sp. showed a minimum ST value of 0.021 N/m, 0.023 N/m, 0.022 N/m and 0.010 N/m respectively followed by BS of PFC-4 *P. fluorescens* isolate, showed a minimum ST values of 0.032 N/m, 0.035 N/m, 0.030 and 0.032 N/m respectively with liquids viz., distilled water, glycerol (10^{-1} diluted), cyclohexane and methoxy ethanol monomethyl ether respectively. BS of other isolates viz., KCC-2, MCN-3, MCC-2, KFN2, KFS1 and DTSC3 also showed a reduction in the ST value of the four liquids tested.

Bioactivity of biosurfactants has been assessed by several workers and their results are also in confirmation with the present findings. Lang *et al.* (1998) reported the trehalose mycolate surfactants of *Rhodococcus erythropolis* in crude form were able to reduce the surface tension of water from 72 Nm^{-1} to 26 Nm^{-1}. Cooper and Goldenberg (1987) studied about two *Bacillus* sp., which produced bioemulsifiers, among these one strain *B. cereus* IAF 346, produced monoglyceride BS and lowered the ST of water to 28 m Nm^{-1}. Several earlier reports have shown that *Pseudomonas* sp. produced extracellular BS and also reduced the ST of water (Phale *et al.*, 1995; Lang and Wulbrandt,1999). Dubey and Juwarker (2004) reported an oily sludge isolate *P.aeruginosa* strain BS2 reduced the surface tension of fermentation broth from 57Nm^{-1} to 27Nm^{-1}, when medium supplemented with glucose and hexadecane as water soluble and insoluble carbon sources respectively.

5.7. EFFECT OF NUTRITIONAL AND CULTURAL CONDITIONS ON PRODUCITON AND EMULSIFICATION ACTIVITY OF BIOSURFACTANTS

The nutritional and the cultural conditions for maximum BS production and emulsification activity varied greatly among the bacterial isolates tested. The bacteria MCN-3, *Geobacillus kaustophilus* gave maximum BS production when mannitol and xylene/neem oil were used as carbon and hydrocarbon sources respectively at pH 7.0 and at incubation temperature of 30 ^{0}C. But maximum emulsification observed, when media were substituted with sucrose and cyclohexane as carbon and hydrocarbon sources respectively, maintained at pH 8.0 and at incubation temperature of 30 ^{0}C. Nazina *et al.* (2001) reported similar thermophilic bacterium *Geobacillus kaustophilus* from high temperature oil fields and grown well at a temperature ranges from 37^{0}C to 68^{0}C and a pH 6.2-7.5.

When isolate KFS1 *Pseudomonas* sp. was tested for BS production and emulsification activity, high BS production was observed when medium substituted with glucose and xylene as carbon and hydrocarbon sources respectively, at pH 8.0 and at temperature of 30 ^{0}C. But emulsification activity was maximum with glucose and cyclohexane as carbon and hydrocarbon source, at pH 7.0 and at 30 ^{0}C.

In the case of DTSC3 *Pseudomonas* sp. isolate, maximum BS production was recorded, when medium was substituted with glucose and neem oil / xylene as carbon and hydrocarbon sources at pH 6.0-8.0 and incubated at 20 ^{0}C. The emulsification activity of the same isolate was found to be high, when medium supplemented with glucose and neem oil/xylene as carbon and hydrocarbon sources, at pH 7.0-8.0 and at 30 ^{0}C incubation temperature.

The isolate KFN2 *Pseudomonas* sp. gave maximum BS production with medium containing mannitol and neem oil as carbon and hydrocarbon sources respectively, maintained at pH 8.0, at 30^{0}C incubation temperature. The emulsification activity of the isolate was found to be maximum under the same conditions favourable for the highest BS production (mannitol, neem oil as carbon and hydrocarbon source, at pH 8.0 and at 30 ^{0}C incubation temperature).

Perusal of literature showed that the yield of BS greatly depended on the nutritional environment of the growing organism. Ruwaida *et al.* (1991b) found that the BS producing bacterium *Rhodococcus* yielded maximum BS production when grown on hydrocarbon as the sole carbon source. Marcin *et al.* (1993) reported that olive oil induced BS production in *Pseudomonas aeruginosa* MB 5001.

Banat (1993) isolated a thermophilic *Bacillus* strain on a hydrocarbon containing medium and which grew at a temperature up to 50 ^{0}C and the BS of this bacterium emulsified kerosene and other hydrocarbons efficiently. The bacterium

Klebsiella oxytoca produced extra cellular BS when grown in a medium with water-soluble carbohydrate as the carbon source and it gave high emulsification activity at pH 7.0, when incubated at 30 ^{0}C (Hwang, 1993). Studies on sugar sources on BS production by *Candida apicola* revealed that large amount of BS sophorolipid was noticed in medium containing glucose or sucrose, and BS production was negligible in maltose added medium (Hommel and Huse, 1993).

Earlier reports revealed that *Pseudomonas* sp. can be grown in a wide range of temperature. The genus *Pseudomonas* which secreted an extracellular lipase on medium, is found to be thermo resistant and very active at alkaline pH and capable of growing in temperature ranging between 15 ^{0}C and 55 ^{0}C (Chavez and Palmeros, 1994). Phale *et al.* (1995) reported that *Pseudomonas maltophilia* produced more amount of extracellular biosurfactant 'Biosur-Pm' at pH 7.0 with less surface hydrophobicity and at pH 8.0, the cells produced less 'Biosur-Pm' with more cell surface hydrophobicity. This revealed that BS of *P. maltophilia* required varying pH for its BS production and emulsifying activity.

Studies revealed that, carbon sources are more effective for BS production than hydrocarbon sources. Deziel *et al.* (1996) compared the BS production of *P. aeruginosa* 195 J by growing them on naphthalene and in mannitol as hydrocarbon and carbon sources respectively. They found that the maximum BS production was obtained in mannitol as carbon source where as it delayed on naphthalene.

Makkar and Cameotra (1998) reported *B. subtilis* when grown at thermophilic (45 ^{0}C) and mesophilic (30 ^{0}C) conditions, produced BS and reduced surface tension of cell free broth. At 30 ^{0}C, the bacterium produced more BS and it was found to be stable at 100 ^{0}C and within a wide pH range (3-11). Vipulanandan and Ren (2000) reported that kerosene and vegetable oil have induced BS production in *Pseudomonas*

sp. Yateem *et al.* (2002) found that BS stimulating carbon source differed with respect to different strains of *P. aeruginosa*.

Syal and Ramamurthy (2003) studied the ability of *Acinetobacter* isolate to produce BS activity using different carbon sources viz., glucose and sucrose respectively. The bacterium grew rapidly and profusely in glucose, but grew rather poorly on sucrose because it was unable to metabolise sucrose rapidly. But the isolate when grown on hydrocarbon showed significant amount of surfactant activity than grown on sugar sources. But the same isolate failed to grow on xylene, so no BS produced in that condition. Shin *et al.* (2004) reported in presence of 240 ppm rhamnolipid, the rate of phenanthrene solubilization varied with wide range of pH from 4.0 to pH 8.0 and highest solubility was detected at a pH of 4.5 to 5.5. They found that apparent solubility of phenanthrene at pH 5.5 was 3.5 times greater than that at pH 7.0.

5.8. STUDIES ON THE DEGRADATION OF PESTICIDES BY BIOSURFACTANT BACTERIA

5.8.1. Degradation of chlorpyriphos

Chlorpyriphos is one of the widely used organophorus insecticides all over the country. In the present study, chlorpyriphos was taken as the test chemical for studying the extent of degradation by BS bacteria. The concentration of chlorpyriphos in the non-enriched soil sample (without bacteria) was 55.84 µg / g soil. In enriched soil samples, chlorpyriphos concentration was found to be declined to 46.42 µg /g, 16.03 µg /g and 55.17 µg /g, due to the bacteria MCN-3 *Geobacillus kaustophilus* (chlorpyriphos treated plot), KFS1 *Pseudomonas* sp. (*Eucalyptus* plantations) and DTSC3 *Pseudomonas* sp. (mancozeb treated vegetable plot) respectively at 40 DAA

(days after application). In the case of KFS1 isolate, the per cent degradation over control was maximum (71.29%) followed by the isolates MCN-3 (16.87%) and DTSC3 (1.2%). This suggested that *Eucalyptus* litter enriched soil having hydrocabon load might have invigorated the development of inherent bacteria capable of producing biosurfactants.

Biodegradation property of BS bacteria can be ideally suited for having a safe, pollution-free soil and environment, especially for removal of the residual toxicity of the applied pesticides, an important step in bioremediation. Survey of the literature revealed that application of BS in the biodegradation of pesticides is still in its infancy, when compared to the cases of application tried in the field of hydrocarbons. The first microorganism that could degrade organophosphorus compounds was isolated by Sethunathan and Yoshida (1973a) and identified as *Flavobacterium* sp. However, unlike other organophosphorus, there is no report of enhanced degradation of chlorpyriphos since its first use in 1965 (Racke *et al.*, 1990). Singh *et al.* (2003) isolated chlorpyriphos degrading bacteria from some of the Australian soils.

In general, the first step in the aerobic bacterial degradation is the hydroxylation of an aromatic ring *via* a dioxygenase, with the formation of a cis–dihydrodiol. The cis-dihydrodiol is then dehydrogenated to give a catechol, which undergoes further ring cleavage and is transformed into intermediates that enter the central pathways of metabolism and are used for energy production and biosynthesis (Mueller *et al.*, 1996). The ability of a microbe to metabolize a compound depends on the presence of inducible enzymes in the cell, which in turn depends on its genetic make up, or mutation and the presence of constitutive enzymes (Torstensson, 1990).

The bacterial degradation of an organophosphorus compound, the first step catalyzed by organophoshate hydrolase or phosphotriesterase enzyme. The organophosphate hydrolase encoding gene opd (organo phosphate degrading) gene

has been isolated from geographically different regions and taxonomically different species. This gene has been sequenced and cloned in different organisms, and altered for better activity (Singh *et al.*, 2004).

5.8.2. Degradation of mancozeb

Perusal of the literature revealed that degradation of fungicides, has not been studied as intensively as that of insecticides. In the present study, mancozeb was used as the test material for the studies on biodegradation by BS bacteria. Mancozeb is one of the members of ethylene bis dithiocarbanate (EBDC) fungicides. Mancozeb hydrolyses in water, and the major degradates are ethylelne thiourea (ETU), Ethylene area (EU) and ethylene bis isothiocyanate sulphide (EBIS). Microorganisam mineralize mancozeb by degrading it to carbon dioxide (Vonk and Kaars, 1976).

Mancozeb residue analysis was carried out at 5, 10, 40 days after application (DAA). The concentration of mancozeb in the non-enriched soil sample was 0.738 µg /g in 5DAA. In enriched soil samples, the concentration of the chemical was reduced to 0.681 µg /g, 0.707 µg /g and 0.677 µg /g per gram soil due to BS bacteria MCN-3 *Geobacillus kaustophilus* (chlorpyriphos treated plot), KFS1 *Pseudomonas* sp. (*Eucalyptus* treated plot) and DTSC3 *Pseudomonas* sp. (mancozeb treated plot) respectively at 5DAA and the per cent reduction over control corresponds to the bacteria were 7.72, 4.20 and 8.26 respectively. At 10DAA, mancozeb residue due to BS bacteria MCN-3, KFS1 and DTSC3 were 0.568 µg, 0.634 µg and 0.541 µg per gram soil respectively, and the per cent reduction over control corresponds to the bacteria were 19.08, 9.69 and 22.93 respectively. At 40 DAA, maximum reduction of mancozeb (0.478 µg /g soil) was obtained due to the BS bacteria DTSC3 (mancozeb treated plot) and the per cent reduction over control was 28.44. These findings revealed that the

bacterium isolated from the mancozeb treated plot is more capable of degrading the particular fungicide as compared to other bacterial isolates.

A perusal of literature showed that informations are available on the degradation of pesticide and herbicide due to various bacteria. Dithiocarbamates constitute the most important class of organic fungicides for plant disease control on a world wide basis. Morrill *et al* (1982) reported that in the soil, the bacteria *Thiobacillus* mediated the decomposition process of thiocarbamic acid derivatives through hydrolysis, demthylation and oxidation process. Xenobiotic compounds like atrazine, 2, 4 – D,dimethoate were degraded by the bacterial strains *viz*, *Bacillus* sp *Arthrobacter* sp *Achromobacter* sp *Pseudomonas* sp and *Rhodococcus* sp respectively (Loos, 1975; Koorpraditskul *et al.*,1993; Saraswat and Gaur,1995; Gowrisankar *et al* 2002). Insectides like lindane and endosulfan were reported to be degraded by the BS bacteria non fluorescent *Pseudomonas* sp. and *Bacillus subtilis* MTCC 1427 respectively (Girija *et al.*, 2000; Awasthi *et al.*, 2003).

5.9. *In vitro* ANTIMICROBIAL ACTIVITY OF SELECTED BS BACTERIA AGAINST SOIL BORNE PATHOGENS

Antimicrobial activity of bacteria varied with different soil borne pathogens. In the present study, the isolate DTSC3 *Pseudomonas* sp. gave maximum per cent inhibition of *Pythium aphanidermatum* followed by MCN–3, *Geobacillus kaustophilus*, DTSC5, *Pseudomonas* sp. and KFS1, *Pseudomonas* sp. But against *Phytophthora capsici*, the six isolates (except KCC–2 *Pseudomonas* sp. and MCC–2 *Pseudomonas* sp.) showed good inhibition property. When *Rhizoctonia solani* screened with the selected BS bacterial isolates, MCN-3 gave maximum inhibition followed by DTSC5, PFC-4 and DTSC3.

Antimicrobial activity of BS bacteria has been reported by several workers. BS bacteria *P.fluorescens* DR 54 showed antagonistic properties against *Pythium ultimum* and *R. Solani* under both *in vitro* and *in planta* conditions. The study highlighted that *P.fluorescens* produced an antibiotic viscosinamide, which has biosurfactant properties that reduced fungal growth and aerial mycelium development of both the pathogens (Nielson *et al.* 1999). Ficke *et al.* (2004) reported that *Pseudomonas* sp produced BS, and used as a potential biocontrol agents of plant pathogenic Oomycetes especially, against *Phytophthora* sp. Rhamnolipid, extracellular metabolities of *P. aeruginosa* with surfactant properties was very effective in controlling the spread of brown rot disease caused by *Phytophthora cryptogea* (Jonghe *et al.* 2005).

5.10. COMPATIBILITY OF SELECTED BS BACTERIA AGAINST BIOAGENTS

Compatibility of the eight BS bacteria were tested with the standard biocontrol agent *P. fluorescens*. Except the two isolates, KCC - 2 and MCN - 3 other six isolates *viz,* MCC-2, KFN2, KFS1, PFC-4, DTSC3 and DTSC5 were found to be compatible with the tested biocontrol agent. The two isolates, KCC - 2 and MCN - 3 showed inhibition zones of 7mm and 8 mm respectively.

Compatibility of these BS bacteria was also tested with *Trichoderma harzianum* and *T. viride,* and all the isolates were found to be compatible to each other. Jisha *et al.* (2002) studied the mutual compatibility of *T. harzianum* and *P. fluorescens* and revealed that they were compatible to each other.

The cullture filtrate of the BS producing bacterial strain *Bacillus subtilis* have showed strong antifungal activity against *T. harzianum* . This is the first report that antifungal compounds produced by *B.subtilis* can induce chlamydospore formation in biocontrol fungi at relatively low concentration (Li *et al.* 2005).

5.11. GROWTH PROMOTING EFFECT OF SELECTED BS BACTERIA

In cowpea seeds, germination per cent ranged from 90 to 100 where as in sorghum seeds, it was 80 to 100. In cowpea seeds, radicle length was maximum (6.020 cm) due to MCN-3 isolate, which was followed by PFC-4 (5.720 cm). However, maximum plumule length (7.220 cm) was noticed due to the treatment with BS bacteria MCN-3, *Geobacillus kaustophilus* that was followed by the isolates PFC-4, MCC-2, DTSC3 and DTSC5 in enhancing plant growth as evidenced by plumule length. In sorghum seeds, radicle length was maximum (3.178 cm) due to the treatment of MCN-3, which was followed by KFS1 and PFC - 4. Maximum plumule length (1.705 cm) was noticed due to MCC-2 isolate, which was followed by isolates MCN-3, PFC-4 and KFN2. Similar work has been reported by Rahman *et al.* (2002) in that, mixed bacterial consortium and rhamnolipid BS produced by *Pseudomonas* sp. were treated in *Phaseolus aureus* Rox B seeds, and significantly improved seed germination, shoot length and root length.

Summing up the findings so far it may be concluded that, the present investigation revealed the prominence or presence of biosurfactant producing bacteria in the soils of Kerala which have got effective emulsification property, surface tension lowering activity of hydrocarbons and also they are promising in the biodegradation of pesticides. Besides these, certain bacteria had antagonistic action against soil borne pathogens and were also compatible with standard biocontrol agents. *In situ* remediation by direct seeding of BS bacteria, use of cheaper substrates for large scale BS production and development of genetically modified microbes with xenobiotic degrading, catabolic genes are the future line of work.

6. SUMMARY

The present study on "Biosurfactant producing bacteria from selected soils of Kerala" was carried out with the objective of selecting efficient bacterial strains for the production of biosurfactants. Biosurfactants (BS) are of increasing interest commercially as substitutes for synthetic surfactants for various industrial and environmental applications.

Soil samples were collected from different hydrocarbon contaminated sites particularly from forest soils, soil polluted with automobile fuel stations, lubricant spill overs, drains of ayurvedic nursing homes, plots of permanent herbicidal and pesticidal trials. A total of ninety two heterotrophic bacteria were isolated from the soils by crude oil and neem oil enrichment technique. Crude oil and neem oil enrichment gave significant increase in bacterial population when compared to control. The soil samples of chlorpyriphos treated oil gave maximum population by both crude oil and neem oil enrichment(124 x 10^7 cfu/g soil and 79.67 x 10^7 cfu/g soil respectively), followed by herbicide treated plot-Location 2 (100.67 x 10^7 cfu/g soil and 74.67 x 10^7 cfu/g soil respectively). But in the soil samples from mancozeb treated vegetable plot (MSV-1) and *Eucalyptus* plantations (EFP-1), the bacterial population were almost same even after enrichment.

Ninety two heterotrophic bacteria obtained from these soil samples were screened for detecting BS producing bacteria by drop collapse assay and xylene spray method. Among this, 36 isolates gave positive results on drop collapse assay. The isolate KFN2 (*Eucalyptus* plantations) recorded maximum area of dispersion (333 mm^2) followed by DTSC3 (324.6mm^2) from mancozeb treated vegetable plot and MCN-3 (310 mm^2) from chlorpyriphos treated plot. Among the 24 isolates that gave positive result in xylene spray method, KFN2 from *Eucalyptus* plantations gave

maximum area of clear zone (961.62mm^2) followed by the isolate DTSC5 from mancozeb treated vegetable plot (854.86mm^2).

Based on the two screening tests, biosurfactant producing bacterial population were estimated from the selected heterotrophic bacterial population, which recorded a wide variation among the soil samples (8.3-47.5%). Mancozeb treated vegetable plot recorded a maximum of 45.4per cent BS bacteria followed by *Eucalyptus* plantations, Peechi and forest lands, Wyanad (37.5per cent in both cases). Eight promising BS bacterial isolates that gave maximum area of dispersion in drop collapse assay and maximum clear zone in xylene spray method selected for further studies are KCC-2 (Kunnathuvalappil ayurveda nursing home), MCC-2 and MCN-3 (chlorpyriphos treated plot), KFS1, KFN2, PFC-4 (*Eucalyptus* plantations), DTSC3 and DTSC5 (mancozeb treated vegetable plot).

The selected BS bacteria were identified based on cultural, morphological biochemical characters and by the standardized colorimetric assay using Hi Assorted Biochemical test kit. Among the eight BS bacteria, six bacteria were identified as, KCC-2 (*Pseudomonas* sp.), MCC-2 (*Pseudomonas* sp), KFS1(*Pseudomonas* sp.), KFN2 (*Pseudomonas* sp.), DTSC3 (*Pseudomonas* sp.) and DTSC5 (*Pseudomonas* sp.). The isolates MCN-3 and PFC-4 were identified by Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh as *Geobacillus kaustophilus* and *Pseudomonas fluorescens* and their accession nos. are MTCC 8517 and MTCC 8518 respectively.

Extraction of BS from cell free culture filtrates of the bacteria revealed that the isolate KFS1 (*Pseudomonas* sp.) from *Eucalyptus* plantations, Peechi registered maximum BS production (7.95g/l) followed by MCN-3 *Geobacillus kaustophilus* (6.45 g/l) and DTSC3 *Pseudomonas* sp (6.20 g/l) isolated from chlorpyriphos treated plot and mancozeb treated plot respectively. Bioactivity of BS bacteria was studied

by the commonly used indices viz., xylene emulsification assay and surface tension measurement of liquids. In xylene emulsification assay, the eight selected BS isolates were recorded a good order of emulsification at 1h, which slightly increased after 24h. This high absorbance at 24h is due to a high level of dispersion of xylene in the buffer. Surface tension (ST) measurement of liquids viz., water, glycerol, cyclohexane and methoxyethanol monomethyl ether after treatment with different biosurfactants, indicated lowering of ST values. BS produced by the isolate DTSC5 *Pseudomonas* sp. (mancozeb treated vegetable plot) recorded a minimum level of ST values in all the four liquids tested followed by the BS of PFC-4 *P. fluorescens* (*Eucalyptus* plantations, Peechi). High emulsification and ST lowering indicated high bioactivity of the surfactants.

The yield of biosurfactant largely depends on the nutritional and cultural conditions of the growing organism. The BS production and activity of the selected BS isolates were tested using different carbon and hydrocarbon sources. Almost all carbon sources gave good BS production. However, maximum yield of BS was recorded by the isolates MCN-3 (*Geobacillus kaustophilus)* and KFN2 (*Pseudomonas* sp) when mannitol used as carbon source where as, glucose was the best carbon source for KFS1 *(Pseudomonas* sp.) and DTSC3 (*Pseudomonas* sp.) isolates. Of the different hydrocarbon sources, xylene, neem oil and cyclohexane were identified as efficient inducers of BS production and xylene emulsification activity of all the bacterial isolates tested. Studies on optimum pH revealed that the isolates KFS1, DTSC3 and KFN2 recorded maximum BS production and activity at pH 8.0 where as, pH 7.0 was found to be optimum for the isolate MCN-3. Effect of temperature on BS production and activity showed that, 30^0C was optimum for all the isolates tested, except for DTSC3 isolate, where maximum BS production was recorded at 20^0C.

Effect of BS bacteria on the degradation of pesticides viz., chlorpyriphos and mancozeb was carried out under *in vivo* condition. Initial concentration of chlorpyriphos was 55.84 µg/g. When enriched with bacterial cultures, the concentration of chlorpyriphos was found to be declined to 46.42 µg/ g, 16.03 µg/ g and 55.17 µg / g due to the BS bacteria MCN-3 (chlorpyriphos treated plot), KFS1 (*Eucalyptus* plantations) and DTSC3 (mancozeb treated plot) respectively at 40 DAA. The per cent degradation of chlorpyriphos over control was found to be maximum in KFS1 enriched soil (71.29 per cent) followed by the isolate MCN-3 (16.87 per cent) and DTSC3 (1.2 per cent) respectively. Effect of degradation of mancozeb due to BS bacteria revealed that concentration of mancozeb was found to be declined at every interval compared to control. The maximum degradation of mancozeb (40DAA) was observed in DTSC3 isolate enriched soil (28.44 per cent) followed by MCN-3 and KFS1 isolates and their per cent of degradation was 23.95 per cent and 14.07 per cent respectively.

Antimicrobial activty of the selected BS isolates against soil borne pathogens revealed that the isolate DTSC3 (*Pseudomonas* sp.) gave maximum inhibition to *Pythium aphanidermatum* which followed by the isolates MCN-3, DTSC5 and KFS1. The six isolates viz., MCN-3, KFS1, PFC-4, KFN2, DTSC3 and DTSC5 showed good percent inhibition against *Phytophthora capsici*. MCN-3 (*Geobacillus kaustophilus)* isolate gave maximum per cent inhibition against *Rhizoctonia solani*. When compatibility of selected BS bacteria tested with *Pseudomonas fluorescens, Trichoderma harzianum* and *T. viride*, the six isolates viz., MCC-2, KFN2, KFS1, PFC-4, DTSC3 and DTSC5 were found to be compatible with the tested biocontrol agents. The isolates KCC-2 and MCN-3 gave small inhibition zone to *P. fluorescens* but were compatible to *Trichoderma harzianum* and *T. viride*.

Growth promoting effect of BS bacteria in sorghum seeds showed that MCC-2 isolate (*Pseudomonas* sp.) gave maximum plumule length and MCN-3 isolate gave maximum radicle length and germination per cent ranged from 80 to 100. Where as in cowpea seeds, maximum plumule and radicle length was observed due to MCN-3 isolate and germination per cent was found to be 90 to 100.

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* Originals not seen

Appendix

Appendix I

Composition of different media used in microbiological studies

Selective medium for TAHB

Glucose	-	5.0 g
Yeast extract	-	3.0 g
Peptone	-	5.0 g
Sodium chloride	-	5.0 g
Tap water	-	1000 ml
Agar	-	15.0 g
pH	-	7.0 -7.5
Trace element solution	-	1 ml

Trace element solution

Zinc sulphate	-	24 mg
Copper sulphate	-	8 mg
Manganese sulphate	–	235 mg
Sodium molybdate	-	200 mg
Boric acid	-	280 mg
Distilled water	-	200 mg

Appendix II

Nutrient agar medium

Beef extract	-	1 g
Peptone	-	5.0 g
Sodium chloride	–	5.0 g

Agar	-	15.0.g
pH	-	7.2 – 7.4

Appendix III

Inorganic salt medium (ISM)

Urea	-	2.80 g
Potassium dihydrogen phosphate	-	0.42 g
Disodium hydrogen phosphate	-	0.42 g
Mag nesium sulphate	-	0.10 g
Calcium chloride	-	0. 02 g
Ferrous sulphate	-	0.001 g
Zinc sulphate	-	70 µ g
Copper sulphate	-	50 µ g
Boric Acid	-	10 µ g
Sodium molybdate	-	10 µ g
Distilled water	-	1000 ml
pH	-	7.0
Glucose	-	0.5 %

Appendix IV

Yeast Extract Glucose Agar Medium

Glucose	-	5.0 g
Yeast Extract	-	3.0 g
Peptone	-	5.0 g
Sodium chloride	-	5.0 g
Distilled water	–	1000 ml
pH	–	7.0

Appendix V

Stains used in microbiological studies

Crystal violet

One volume saturated alcohol solution of crystal violet in four volumes of 1 per cent aqueous ammonium oxalate.

Gram's iodine

Iodine crystals	-	1.0 g
Potassium iodide	-	2.0 g
Distilled water	–	300 ml

Safranin

Safranin O	-	0.25 g
Ethanol (95%)	-	10.0 ml
Distilled water	-	100 ml

Dissolve safranin in ethanol and then in water and filter.

Malachite green

Malachite green	-	5.0 g
Distilled water	–	100 ml

AppendixVI

Composition of different media used in microbiological studies

Simmon's citrate agar

Sodium citrate	-	0.2 g
Magnesium sulphate	-	0.02 g
Ammonium dihydrogen phosphate	-	0.1 g
Dipotassium hydrogen phosphate	-	0.1 g
Sodium chloride	-	0.5 g
Bromothymol blue	-	0.008 mg
Agar	-	2.0 g
Distilled water	-	1000.0 ml
pH	-	6.8

AppendixVII

Sulphide indol motility agar medium

Dextrose	-	5.0 g
Peptone	-	7.0 g
Potassium phosphate	-	5.0 g
Distilled water	-	1000 ml
Agar	-	15 g
pH	-	6.9

Appendix VIII

Tryptone glucose broth

Tryptone	-	1.0 g
Glucose	-	1.0 g
Distilled water	-	1000 ml

Appendix IX

Thornley's medium

Peptone	–	1.0 g
K_2HPO_4	–	0.3 g
NaCl	–	5 g
Agar	–	3 g
Phenol red	–	0.01 g
L – Arginine monochloride	–	10.0 g
Distilled water	–	1000 ml
pH	–	7.2

Appendix X

Nitrate broth medium

KNO_3 (Nitrate free)	–	1.0g
Peptone	–	10.03 g
Beef extract	–	5 g
Distilled water	–	1000 ml
pH	–	7.0

Appendix XI

King's B medium

Peptone	-	2.0 g
Glycerol	-	1.0 g
K_2HPO_4	–	0.15 g
$MgSO_4$	-	0.15 g
Distilled water	–	100 ml
Agar	-	2.0g
pH	-	7.2 –7.4

Appendix XII

Potato Dextrose Agar medium

Peeled potato	-	200.0 g
Dextrose	-	20.0 g
Agar	-	20.0 g
Water	-	1000 ml
pH	-	6.0 –6.5

BIOSURFACTANT PRODUCING BACTERIA FROM THE SELECTED SOILS OF KERALA

By

REMYA V. M.

ABSTRACT OF THE THESIS

Submitted in partial fulfilment of the requirement for the degree of

Master of Science in Agriculture

Faculty of Agriculture
Kerala Agricultural University

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ABSTRACT

A study on "Biosurfactant producing bacteria from the selected soils of Kerala" was conducted at College of Horticulture, Vellanikkara during the period from 2004-2006. Eight promising BS producing bacteria were selected by screening a total of 92 heterotrophic bacteria isolated from ten different hydrocarbon contaminated soil samples. Based on the morphological and biochemical characters, out of the 8 selected isolates, six cultures were tentatively identified as KCC-2 (*Pseudomonas* sp.), MCC-2 (*Pseudomonas* sp.), KFS1 (*Pseudomonas* sp.), KFN2 (*Pseudomonas* sp.), DTSC3 (*Pseudomonas* sp.) and DTSC5 (*Pseudomonas* sp.). The other two bacterial isolates MCN-3 and PFC-4 were identified through Microbial Type Culture Collections (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, as *Geobacillus kaustophilus* and *Pseudomonas fluorescens* with accession numbers MTCC 8517 and MTCC 8518 respectively.

Per cent Biosurfactant producing bacterial population was estimated from the selected heterotrophic bacterial population, based on drop collapse assay and xylene spray method. Mancozeb treated vegetable plot gave maximum per cent BS bacteria (45.4per cent) followed by *Eucalyptus* plantations, Peechi and forest lands, Wyanad (37.5per cent in both cases). Extraction of BS production indicated that the isolate KFS1 from *Eucalyptus* plantations, recorded maximum BS production (7.95g/l) followed by MCN-3 (6.45 g/l) and DTSC3 (6.20 g/l). The eight selected BS bacteria recorded good xylene emulsification property. These isolates also lowered the surface tension values of the liquids viz., distilled water, glycerol (10^{-1} dilution), cyclohexane and methoxy ethanol monomethyl ether. The optimum nutritional and cultural conditions required for maximum BS production and emulsification activity were varied greatly among the bacterial isolates tested.

Effect of selected BS bacteria on the degradation of pesticides viz., chlorpyriphos and mancozeb were studied, the isolate KFS1 from *Eucalyptus* plantations reduced the concentration of chlorpyriphos from 55.84 µg /g to 16.03 µg/g soil at 40 days after application (DAA) with 71.29 per cent degradation compared to control. And in mancozeb residue analysis studies, the isolate DTSC3 enriched soil sample, reduced the concentration of mancozeb from 0.738µg/g to 0.478 µg/g soil at 40 DAA and the per cent degradation was 28.44 per cent compared to control.

Among the eight BS isolates, most of them showed high per cent inhibition to soil borne pathogens like *Pythium aphanidermatum, Phytophthora capsici* and *Rhizoctonia solani* and all of them are compatible to *Trichoderma harzianum* and *T. viride.* Among the eight isolates, six were compatible to *Pseudomonas fluorescens.* Selected BS isolates showed plant growth promoting effect by enhanced seed germination, plumule and radicle length in cowpea and sorghum seeds.